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Investigating the impact of ovarian carcinoma ascites on Toll-like receptor mediated dendritic cell activation

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**Thesis submitted for the degree of
Doctor of Philosophy
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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so we may fear less.”

- *Marie Curie*

Abstract

In this study, we investigate the impact of ovarian carcinoma associated ascites on dendritic cell (DC) activation by Toll-like receptor (TLR) agonists *in vitro*. DC have the potential to instigate a tumour-specific adaptive immune response, but their ability to induce differentiation of naïve lymphocytes into effector cells in lymphoid tissues is dependent on their activation status. Here, we examine whether DC activation by TLR agonists is impeded by ovarian carcinoma environment and if so, how these effects can be alleviated.

Our results show that ascites reduces the TLR-mediated up-regulation of the co-stimulatory molecule CD86 and partially inhibits the production of the pro-inflammatory cytokines interleukin-6 (IL-6), IL-12 and tumour necrosis factor α (TNF α) in monocyte-derived DC from healthy donors. We further observe an impaired T cell stimulatory capacity of monocyte-derived DC upon activation with TLR agonists in the presence of ascites, indicating that their function as antigen-presenting cells is affected by the immunosuppressive factors. Selective neutralization of IL-10 and prostaglandin E₂ (PGE₂) *in vitro* alleviates the suppressive effects of ovarian carcinoma associated ascites. However, our results show that autocrine IL-10 contributes to the observed suppression. The role of autocrine PGE₂ is yet unclear, as we have no indication that this protein is produced by TLR-activated monocyte-derived DC. We have established and present here an elegant method to dissect the relative contributions of ascites-derived versus autocrine IL-10 and PGE₂, and experiments to this effect are ongoing.

The findings of this study can enhance the understanding of the ovarian carcinoma environment and its influence and relevance in the context of DC-based vaccines and other immunotherapeutic intervention strategies in ovarian carcinoma.

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Abbreviations

AA = arachidonic acid

APC = antigen presenting cell

APC = allophycocyanin

Arg-1 = arginase-1

Batf3 = Basic leucine zipper transcription factor ATF-like 3

BCR = B cell receptor

BDCA = blood dendritic cell antigen

BIP = binding immunoglobulin protein

BRCA 1 = breast cancer gene 1

BRCA 2 = breast cancer gene 2

CCL = CC chemokine ligand

CCR = CC chemokine receptor

CD = cluster of differentiation

CFSE = carboxyfluorescein succinimidyl ester

CLR = C-type lectin receptor

Clec9A = C-type lectin domain family 9 member A

COX-1 = cyclooxygenase-1

COX-2 = cyclooxygenase-2

CpG = Cytosine-phosphate-Guanine

CRE-AP = cAMP response element adapter protein

CTL = cytotoxic T lymphocyte

CTLA-4 = cytotoxic T lymphocyte antigen-4

DAMP = damage associated molecular patterns

DC = dendritic cell

DMSO = dimethyl sulfoxide

DNA = deoxyribonucleic acid

DNDR-1 = dendritic cell natural killer lectin group receptor 1
dsRNA = double stranded ribonucleic acid
EDTA = ethylene-diamine-tetraacetic acid
ELISA = enzyme-linked immunosorbent assay
FACS = fluorescence activated cell sorting
FCS = fetal calf serum
FIGO = Fédération Internationale de Gynécologie et d'Obstétrique
FITC = fluorescein isothiocyanate
FoxM1 = forkhead box protein M1
FoxP3 = forkhead box P3
GM-CSF = granulocyte-macrophage colony-stimulating factor
HGSC = high-grade serous carcinoma
HIV = human immunodeficiency virus
HMGB1 = high mobility group box 1
IDO = indoleamine 2,3-dioxydase
IFN = interferon
Ig = immunoglobulin
IL = interleukin
IRAK = IL-1 receptor associated kinase
IRF3 = interferon-regulatory factor 3
IRF7 = interferon-regulatory factor 7
KO = knock-out
LIF = leukemia inhibitory factor
LT = lymphotoxin
LPS = lipopolysaccharide
MAP = mitogen-activated protein
MCP-1 = monocyte chemoattractant protein 1
mDC = myeloid dendritic cell

MDSC = myeloid derived suppressor cells

MFI = mean fluorescence intensity

MHC = major histocompatibility complex

MLR = mixed leukocyte reaction

MyD88 = myeloid differentiation factor 88

Nectin2 = nectin-like molecule 2

NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells

NK cell = natural killer cell

NOD = nucleotide-binding oligomerisation domain

NSAID = non-steroidal anti-inflammatory drugs

OD = optical density

ODN = oligodeoxynucleotide

PAMP = pathogen associated molecular pattern

PBMC = peripheral blood mononuclear cells

PBS = phosphate-buffered saline

PD-1 = programmed cell death protein-1

PD-L1 = programmed cell death 1 ligand 1

PD-L2 = programmed cell death 1 ligand 2

pDC = plasmacytoid dendritic cell

PE = phycoerythrin

PE-Cy7 = phycoerythrin conjugated with cyanine 7

Per-CP Cy5.5 = peridin-chlorophyll-protein complex conjugated with cyanine 5.5

PGE₂ = prostaglandin E₂

poly I:C = polyinosinic:polycytidylic acid

PRR = pattern recognition receptor

R848 = resiquimod

RA = rheumatoid arthritis

RIG-I = retinoic acid inducible gene I

RNA = ribonucleic acid
ROS = reactive oxygen species
RT = room temperature
SLE = systemic lupus erythematosus
ssRNA = single stranded ribonucleic acid
STAT3 = signal transducer and activator of transcription 3
TAA = tumour associated antigen
TAM = tumour associated macrophages
TAP = transporter associated with antigen processing
TCR = T cell receptor
TGF β = transforming growth factor β
T_H cell = T helper cell
TIL = tumour infiltrating lymphocytes
TIR = Toll/interleukin-1 receptor
TLR = Toll-like receptor
TNF α = tumor necrosis factor α
T reg = regulatory T cell
TRIF = TIR-domain containing adapter-inducing interferon β
TRAF = TNF α receptor associated factor
VEGF α = vascular endothelial growth factor α

Chapter 1

Introduction

While the foremost function of the immune system is the protection of the host from infectious pathogens, immune malfunction can bear detrimental consequences for the host that stretch beyond the scope of infectious diseases. Challenges lurk not only in the environment in the form of invading pathogens, danger can often arise from within an organism in form of cancerous cells. The immune system has the ability to eliminate tumour cells and this capacity is increasingly gaining relevance and importance and is being intensively explored in the field of cancer immunology and immunotherapy.

The immune system constitutes a large number of cells and molecules that act together to protect the host from disease. In essence, regardless of the nature of the challenge, a functional immune response that leads to host protection requires activation of leukocytes, the cellular constituents of the immune system. The first line of defence is provided by the innate immune system comprising several cell types with diverse functions but the common ability to react to challenges rapidly, together leading to the subsequent initiation of the adaptive immune response. Dendritic cells (DC) are considered pivotal to the linking of the innate and adaptive immune response, and these cells were the primary focus of our study. To appreciate their function in the context of an immune response as a whole, an understanding of the processes and components of the innate and adaptive immune system is necessary.

Measures needed for elimination of cancerous cells resemble in many aspects those required for the elimination of pathogens. However, important differences between the challenge with pathogens versus tumour cells prevail. With an understanding of these differences, it becomes apparent that the immune system has primarily evolved to

protect the host from infections and at the same time, reasons why it so often fails at conquering cancer become evident.

1.1 Pattern recognition

In 1989, Charles Janeway first proposed the existence of evolutionarily conserved molecules essential to pathogen function which are absent from the host but can be recognized by specialized pattern-recognition receptors (PRR) present on host cells (Janeway, 1989). The so-called pathogen-associated molecular patterns (PAMP) are abundant in bacteria and comprise cell wall components such as lipopolysaccharide (LPS), lipoproteins or peptidoglycans, and also bacterial DNA containing unmethylated CpG motifs. Yeast and fungal cell walls have PAMP in the form of mannans and β -glucans, and viral PAMP include single-stranded (ss) and double-stranded (ds) ribonucleic acid (RNA) (Reis e Sousa, 2001).

Upon invasion of the host with pathogens, PAMP act as ‘alarmins’ and can be recognized by several classes of PRR which have been identified to date, including Toll-like receptors (TLR), C-type lectin receptors (CLR) and cytoplasmic PRR such as nucleotide-binding oligomerisation domain (NOD)-like receptors and retinoic acid inducible gene (RIG)-I (Meylan et al., 2006; Diebold, 2009). Each PRR recognizes only certain PAMP, and the various PRR are differentially expressed on leukocytes including monocytes, macrophages and DC which are all components of the innate immune system. These cells thereby possess the ability to sense invading pathogens in their surroundings.

In contrast to pathogens, tumour cells constitute the host’s very own cells and do not contain PAMP. Hence, although leukocytes may be present in immediate tumour

surroundings, cancerous cells do not trigger their PRR. This is a crucial difference that distinguishes an infectious challenge from a challenge posed by a tumour.

1.1.1 Toll-like receptors

The best characterized family of PRR are TLR, which had originally been discovered in the drosophila where they were shown to play an important role in the immune response against fungal pathogen (Stein et al., 1991; Lemaitre et al., 1996). The first human TLR was identified by Ruslan Medzhitov and Charles Janeway, who have also demonstrated that the triggering of TLR can induce expression of cytokines and co-stimulatory surface molecules (Medzhitov et al., 1997).

To date, 10 TLR have been described in the human system. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell surface, allowing the cell to directly sense surrounding material through these receptors. In contrast, TLR3, TLR7, TLR8 and TLR9 are located intracellularly, more specifically on the endosomal membrane. Consequently, ligands of the TLR found on the cell surface include molecules that are frequently found on extracellular pathogens, such as LPS which triggers TLR4 or the bacterial protein flagellin which is recognized by TLR5 (Poltorak et al., 1998; Hayashi et al., 2001). Endosomal TLR specialize in detection of nucleic acids. TLR3 is triggered by dsRNA (Alexopoulou et al., 2001), while ssRNA is a ligand of TLR7 and TLR8 (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004) and TLR9 recognizes bacterial (Hemmi et al., 2000) or viral deoxyribonucleic acid (DNA) (Lund et al., 2003). The access of such nucleic acids to endosomal TLR is provided either by direct infection of a cell in case of non-fusogenic viruses and bacteria that enter cells by receptor-mediated uptake, or by uptake of pathogen material by phagocytosis.

Recognition of ligands is mediated by the extracellular or endosomal domain of the TLR, and signals are transduced by a number of cytoplasmic molecules. The

Toll/interleukin-1 receptor (TIR) domain of the TLR associates with the TIR domain of adaptor protein myeloid differentiation factor 88 (MyD88) and via a variety of signalling cascades involving, among others, adaptor TNF α receptor associated factor (TRAF), IL-1 receptor associated kinase (IRAK) and mitogen-activated protein (MAP) kinase, this leads to activation of transcription factor NF κ B. This results in production of an array of pro-inflammatory proteins (Takeda and Akira, 2004).

The MyD88 pathway is triggered by all 10 TLR with the exception of TLR3. This endosomal TLR signals via a MyD88-independent pathway mediated by TIR-domain containing adapter-inducing interferon β (TRIF). The resulting signalling cascade can either result in NF κ B activation, or in stimulation of interferon-regulatory factor 3 (IRF3), a transcription factor that promotes transcription of the gene for interferon β (IFN β) (Takeda and Akira, 2004).

TLR are differentially expressed on many immune cells, and notably on cells of the innate immune system including antigen presenting cells (APC). Triggering of TLR induces innate immune activation, which is a pre-requisite for the instigation of an adaptive immune response.

<u>Toll-like receptor</u>	<u>Location</u>	<u>Key ligands</u>	<u>Adapter molecules</u>	<u>Signalling pathway</u>
TLR1	Cell surface	Lipids, Peptidoglycan	MyD88, TIRAP	NF- κ B, CRE-AP-1
TLR2	Cell surface	Lipids, Peptidoglycan	MyD88, TIRAP	NF- κ B, ROS, CRE-AP-1
TLR3	Intracellular	dsRNA	TRIF	NF- κ B, IRF3, ROS
TLR4	Cell surface	LPS	MyD88, TRIF, TRAM, TIRAP	NF- κ B, IRF3, CRE-AP-1
TLR5	Cell surface	Flagellin	MyD88	NF- κ B, CRE-AP-1
TLR6	Cell surface	Lipids, Peptidoglycan	MyD88, TIRAP	NF- κ B, CRE-AP-1
TLR7	Intracellular	ssRNA	MyD88	NF- κ B, IRF7, CRE-AP-1
TLR8	Intracellular	ssRNA	MyD88	NF- κ B, IRF7, CRE-AP-1
TLR9	Intracellular	CpG-ODN	MyD88	NF- κ B, IRF7, CRE-AP-1
TLR10	Cell surface	?	MyD88	NF- κ B, CRE-AP-1

Table 1.1 Human TLR

Overview of human TLRs and their localisation within the cell, key ligands, adapter molecules and triggered signalling cascades.

1.2 Innate immune activation

The innate immune system comprises several cell types which are either found in circulation or distributed throughout tissues of the body. Macrophages are tissue resident phagocytes with a prominent ability to take up material from their surroundings. They differentiate from monocytes, which are found in circulation, constituting approximately 10% of peripheral blood mononuclear cells (PBMC). Upon reception of chemotactic signals conveyed by chemokines such as monocyte chemoattractant protein 1 (MCP-1) (CCL2), monocytes leave the blood stream by extravasation (Matsushima et al., 1989). Their differentiation and polarization into macrophages is influenced by their surroundings. Monocytes express TLR and in the presence of pathogens and PAMP in the tissue, they differentiate into the ‘classical’ M1 type macrophages. These cells are very efficient at engulfing and eliminating pathogens and infected cells. M1 equally express a range of PRR, and upon encounter and uptake of infectious material, these receptors are triggered, leading to M1 activation which is characterized by up-regulation of surface molecules such as major histocompatibility complex (MHC) class II and co-stimulatory molecules CD80, CD86 and others. Although macrophages possess the ability to process and present antigen to T cells, this function is secondary. Primarily, production and secretion of pro-inflammatory cytokines by M1 macrophages upon PAMP recognition via PRR contributes to the orchestration of the local immune response, promoting a pro-inflammatory environment by activation and recruitment of further leukocyte subsets.

The monocyte attractant MCP-1 is commonly found in tumour tissue, leading to an infiltration of tumours with monocytes. However, in absence of infection and presence of tumour-derived factors such as IL-10, monocyte differentiation is skewed towards the ‘alternative’ M2 macrophage type. These cells differ considerably from M1 macrophages both in their phenotype and functions (Mantovani et al., 2002). By

secretion of prostaglandins and immunosuppressive cytokines IL-10 and transforming growth factor β (TGF β), they heavily contribute to intratumoral immunosuppression and impair activation and functionality of surrounding leukocyte subsets. In the context of tumours, M2 macrophages are referred to as tumour associated macrophages (TAM). TAM have emerged as an important negative prognostic factor in many human cancers, and their characterization is therefore of great interest in the field of cancer immunology and immunotherapy (Bingle et al., 2002; Sica et al., 2006; Heusinkveld and van der Burg, 2011).

In many ways, the tumour environment resembles a site of infection, abundant in soluble mediators, with increased vascular permeability and swelling. However, a crucial difference is the absence of pathogens. In absence of infection, inflammatory responses in tissue are referred to as sterile inflammation. The triggers of sterile inflammation in tumours are complex and include mediators produced by tumour cells, stroma and infiltrating leukocytes, as well as apoptotic cells (Chen and Nunez, 2010)

The contrasting roles of M1 and M2 macrophages illustrate that the underlying cause of inflammation can gravely influence the immune response. The presence of PAMP at a site of infection promotes the differentiation of monocytes into pro-inflammatory M1 macrophages, while the absence of pathogens during sterile inflammation together with presence of tumour-derived factors favours the induction of immunosuppressive TAM.

Differences between sterile and infection-triggered inflammation extend to other cells of the innate immune system including neutrophils, natural killer cells (NK cells) and circulating monocytes, which all have the ability to sense invading pathogens via PRR. Collectively, the processes of the innate immune response shape the nature of the successive adaptive immune response. Through their compelling antigen presenting ability, DC of the innate immune system have emerged as a pivotal and direct link between innate and adaptive immunity and their activation is therefore of paramount

importance for the instigation of an antigen-specific adaptive immune response, both in infection and cancer.

1.2.1 Dendritic cells

The identification of DC by Ralph Steinman and Zanvil Cohn in 1973 was a landmark discovery in modern immunology (Steinman and Cohn, 1973). Steinman described DC as a sparse population of murine spleen leukocytes with an exceptional ability to potently stimulate proliferation of lymphocytes in mixed leukocyte reactions (MLR). He coined the name ‘dendritic cell’ due to the tree-like morphology of the cells’ cytoplasmic protrusions. After the initial discovery, several publications outlining their morphological and functional characteristics followed in short succession (Steinman and Cohn, 1974; Steinman et al., 1974; Steinman et al., 1975). Over several years, methods to isolate and culture DC were established and optimized, but working with this cell type nevertheless proved challenging due to the intricacy of isolation methods as well as paucity of DC among leukocytes, further complicated by the complexity of DC subsets. In 1982, DC were discovered in human peripheral blood (Van Voorhis et al., 1982), but their study was hindered by the same technical and methodical difficulties as previously encountered in the murine system. DC comprise approximately 1% of PBMC, and hence the numbers obtainable from human subjects are very limited. A milestone in the research of human DC was the finding that DC could be generated *in vitro* from the considerably more populous monocytes with the help of recombinant growth factors granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 (Sallusto and Lanzavecchia, 1994). Termed monocyte-derived DC, these cells have proven to be an extremely useful tool for the study of human DC. In addition, over the last decade, simple and efficient methods for isolation of primary DC have become commercially available, although their sparse numbers do continue

to pose a challenge. Nevertheless, after 40 years of DC research, the field of immunology holds an accomplished understanding of the functions and characteristics of human DC subsets.

Three populations of human DC can be distinguished in peripheral blood based on their developmental pathways and functionality. All are derived from the common myeloid progenitor in the bone marrow, and during development, guided by a myriad of distinct cytokines and transcription factors, they differentiate into different subsets.

1.2.1.1 Myeloid DC

Human myeloid DC (mDC) are considered the body's most efficient APC, positioned at the interface between innate and adaptive immunity. mDC are phenotypically characterized by expression of high levels of MHC class II, and two subpopulations can be distinguished by differential expression of further phenotypic markers. The mDC1 subset typically expresses blood dendritic cell antigen (BDCA)-1 and, constituting approximately 0.5% of PBMC, is considerably more frequent than mDC2. High expression levels of BDCA-3 distinguish mDC2, an extremely rare cell type, representing <0.1% of PBMC (Dzionek et al., 2000).

mDC express PRR for the recognition of PAMP. More specifically, peripheral blood mDC1 express high levels of TLR2 and TLR5 and low levels of TLR4, TLR6 and TLR8 (Jarrossay et al., 2001), while mDC2 were shown to express high levels of TLR1, TLR3, TLR6 and TLR10, intermediate levels of TLR2 and low levels of TLR8 (Jongbloed et al., 2010; Poulin et al., 2010). In addition, both subsets harbour PRR of other classes such as CLR and cytoplasmic PRR, and mDC are thus well equipped to sense a multitude of invading pathogens. In the steady state, mDC reside in peripheral tissues and survey the environment for potential incoming infectious agents. Upon encounter and uptake of pathogen material, triggering of PRR induces mDC activation,

which is characterized by up-regulation of the chemokine receptor CCR7, leading to increased migratory ability of the cells. Via chemotactic signals conveyed by CCR7 ligands CCL19 and CCL21, activated, antigen-loaded mDC travel via lymphatic vessels to local lymph nodes (Forster et al., 2008). Apart from up-regulation of CCR7, activation of mDC is characterized by increased expression levels of MHC II and co-stimulatory molecules such as CD80 and CD86, as well as secretion of an array of cytokines including IL-6, IL-12, TNF α and others (Reis e Sousa, 2001). In lymphoid tissues, mDC present complexes of antigen peptides and MHC molecules to T lymphocytes, which sample these complexes via T cell receptors (TCR). Upon recognition of a cognate MHC-peptide complex via TCR, T cells undergo differentiation and clonal expansion into effector T cells.

DC activation as triggered by PRR due to presence of PAMP in an infectious scenario is paramount to efficient T cell priming and instigation of an antigen-specific adaptive immune response. This is supported by reports showing that in the steady state, small numbers of DC constantly take up host cellular material from dying cells in the periphery and travel to the lymph nodes, and these cells do not stimulate autoimmune T cell responses but instead, they promote tolerance (Reis e Sousa, 2006). In contrast, uptake of cellular material from infected cells that contain PAMP leads to immunity.

Scavenging of apoptotic cell material is a function fulfilled in particular by mDC2. This has been elucidated only recently, when this subtype was recognized as an equivalent of murine CD8 α^+ DC (Jongbloed et al., 2010; Poulin et al., 2010) which have long known to be specialized in uptake of cell-associated antigens (Iyoda et al., 2002). The studies by Jongbloed et al. and Poulin et al. demonstrate that phenotypically, BDCA3 $^+$ mDC2 resemble murine CD8 α^+ DC, as suggested by the expression of high levels of the C-type lectin receptor DNGR1 (Clec9A), adhesion protein Necl2 as well as TLR3, and further the transcription factor Batf3 which had previously been identified as a defining factor for the developmental lineage of CD8 α^+ DC in mice (Hildner et al., 2008). Interestingly, the authors of the study by

Poulin et al. have established a protocol for the *in vitro* generation of BDCA3⁺ mDC2 from CD34⁺ hematopoietic stem cells from human cord blood. In addition, they show that primary BDCA3⁺ mDC2 can be isolated from spleen suspensions of humanized mice (Poulin et al., 2010). Both these methods could help facilitate research with this extremely rare cell type and its properties and functions could thus be elucidated further in the near future.

So far, these studies have shown that human BDCA3⁺ mDC2 are efficient at uptake of exogenous material which they subsequently cross-present to CD8⁺ cytotoxic T cells. Cross-presentation is the ability to process exogenous antigens in such a way that they are presented on MHC class I molecules where they can be recognized by CD8⁺ T lymphocytes. Typically, only peptides from endogenous antigens that are synthesized within the cell, such as products of viruses that have infected the cell and hijacked its protein synthesis machinery are presented on MHC class I molecules. In contrast, exogenous antigen taken up by an APC is processed in a different manner and is loaded onto MHC class II molecules. Complexes of exogenous peptides and MHC class II molecules are recognized by CD4⁺ T helper cells (T_H cells) (Heath and Carbone, 2001).

All nucleated cells express MHC class I on their cell surface (Swain, 1983), whereas expression of MHC class II is restricted to APC. Presentation of endogenous antigens coupled to MHC class I and their recognition by CD8⁺ T cells is a useful mechanism in viral infection. By direct presentation, virus infected cells can be recognized and destroyed by cytotoxic T lymphocytes (CTL). However, only professional APC such as mDC have the ability to potently prime an immune response leading to a substantial population of antigen-specific cytotoxic T cells, but infected mDC may be compromised in their functionality (Gabrilovich et al., 1994; Fugier-Vivier et al., 1997). Moreover, not all viral pathogens infect mDC. Under such circumstances, the ability of specialized DC subsets to cross-present exogenous antigens is required (Bevan, 1987). While it is now understood that other DC subsets also have the

capacity to cross-present soluble or particulate antigen (Pooley et al., 2001), murine CD8 α^+ DC and human mDC2 are specialized in cross-presentation of cell-associated antigens.

Tumour associated antigens (TAA) constitute cell-associated antigens, and they can equally be processed and cross-presented by these cell types. Similarly to viral infections, cellular immunity is desired in the context of cancer for elimination of tumour target cells. However, due to absence of PAMP associated with scavenged tumour cell material and therefore lack of PRR activation, cross-priming DC typically fail to elicit TAA-specific CTL responses.

In immunotherapeutic concepts that aim to harness the antigen presenting ability of mDC subsets, the use of TLR agonists therefore represents an attractive approach. In essence, application of synthetic PAMP targeting specific TLR receptors mimics an infectious scenario. In the context of cancer immunotherapy, use of TLR agonists can thus lead to mDC activation, enabling efficient TAA (cross-)presentation and instigation of tumour specific adaptive immune responses.

Not only direct activation of mDC subsets via PRR is, however, paramount to anti-tumour immunity. Soluble mediators provided by leukocyte subsets other than mDC are required for efficient cross-presentation of TAA and functionality of CTL (Kolumam et al., 2005; Le Bon et al., 2006). One such example was demonstrated in a previous study in our laboratory. In an experimental mouse B16 melanoma pseudometastasis model, our colleagues have shown that type 1 interferon is crucial for the adjuvant activity of TLR7 agonist polyUs21 and, hence, an efficient anti-tumour response (Rajagopal et al., 2010). They could further show that this type 1 interferon was provided by plasmacytoid DC (pDC), which constitute a DC subset distinct from mDC found both in mice and humans.

1.2.1.2 Plasmacytoid DC

Before their classification within the DC lineage and nomenclature as pDC, this cell subset was commonly referred to as interferon-producing cells (Cella et al., 1999; Siegal et al., 1999). pDC in human peripheral blood are characterized by the expression of the surface markers BDCA-2 and -4, as well as IL-3R α (CD123), while they lack the surface marker CD11c which is typically expressed by mDC (Dzionek et al., 2000). Their ability to produce large amounts of type 1 interferons in response to microbial challenge had been recognized early on, and they were postulated to be of considerable importance in the defence against viral infections. Type 1 interferons promote the induction of T_H1 immune responses, enhance cytotoxic T cell responses and play a role in immunoglobulin class switching in B cells (Cousens et al., 1999; Le Bon et al., 2001; Le Bon et al., 2006). The role of pDC in antigen presentation is somewhat controversial, with some reports indicating that they have the ability to present exogenous antigen and can induce tolerance to self-antigens (Kuwana et al., 2001). Other studies suggest that they, in fact, fail to present exogenous antigens but are able to present endogenous antigens such as when directly infected with virus, and that they have the ability to prime cytotoxic T cell responses (Fonteneau et al., 2003; Krug et al., 2003; Salio et al., 2004). pDC could therefore be of importance in viral infections not only due to their type 1 interferon producing capacity, but also as functional APC that can prime virus-specific T cells.

Human pDC express high levels of TLR7 and TLR9, as well as low levels of TLR1, TLR6 and TLR10 (Hornung et al., 2002). It is not well studied and therefore unclear how ligands of TLR1, TLR6 and TLR10 effect pDC. However, it has been well established that TLR7 and TLR9 can be triggered by viral genomic RNA and DNA, respectively, resulting in potent production of type 1 interferon (Krug et al., 2001; Lund et al., 2003; Diebold et al., 2004; Heil et al., 2004). The synthetic compound imiquimod leads to type 1 interferon production via TLR7 stimulation in pDC, and is used for

treatment of basal cell carcinoma (Urosevic et al., 2005; Ghafouri-Fard, 2012). pDC have further been identified in the microenvironment of several other cancers (Zou et al., 2001), and harnessing their potential to enhance potent cellular immunity is increasingly being considered as an attractive concept in cancer immunotherapy (Schettini and Mukherjee, 2008).

The importance of DC as APC directly linking innate and adaptive immunity reaches beyond their antigen processing and presenting abilities. The interaction between DC and the T lymphocytes is complex and requires more than the mere display of peptide-MHC complex on the APC surface and its recognition by cognate TCR. Additional signals are required to facilitate antigen-specific T cell expansion, some of which are DC-derived. These signals are important in the instruction and tailoring of the effector functions of the induced adaptive immune response.

1.3 Adaptive immune activation

Lymphocytes are the cellular components of the adaptive immune system. They originate from the common lymphoid progenitor in the bone marrow, and are divided into two subtypes – the B lymphocyte and T lymphocyte, commonly referred to as B cells and T cells. Both can be found in circulation and in lymphoid tissues either as naïve lymphocytes or, after encountering their specific antigen and differentiation in the course of an adaptive immune response, as effector lymphocytes.

Depending on the type of challenge a host is faced with, different effector functions are required. Infections with extracellular pathogens such as bacteria necessitate primarily the initiation of humoral immunity to facilitate their opsonisation and neutralization, while viral infections call for cellular immunity which leads to recognition and elimination of infected target cells.

1.3.1 B lymphocytes

B lymphocytes are mediators of the adaptive humoral immune response. Through a unique B cell receptor (BCR) displayed on their cell surface, naïve B cells have the ability to recognize and bind antigens freely available in tissues, such as viral particles, bacterial cells or fragmented components of invading pathogens. The BCR represents a membrane-bound immunoglobulin which transmits signals to the cell's interior upon binding of cognate antigen. With the help of interactions with T_H cells, B cells differentiate into plasma cells which specialise in the production and secretion of large amounts of immunoglobulin molecules. Several classes of immunoglobulins have been identified, termed IgA, IgD, IgE, IgG, and IgM. They have different roles in the immune system, and while IgM is the antibody class secreted most rapidly in the time course of an immune response, in a mechanism referred to as 'class switching',

B lymphocytes have the ability to switch to the production of other immunoglobulin classes (Pan-Hammarstrom et al., 2007). Defects of humoral immunity have dramatic impact on the immune competence of a host, making them particularly susceptible to bacterial infections (Fleer, 2000).

Upon binding of antigen via BCR, B cells can act as APC and internalize, process and display peptide-MHC class II complexes for recognition by $CD4^+$ T_H cells on their cells surface. Despite their ability to present antigens to T lymphocytes, the exact role and relevance of B cells as APC in the priming of naïve T cells is not clear. Their importance in orchestration of T cell responses is nevertheless undisputed, and B cell depletion in experimental models leads to disruption of T cell responses such as impaired $CD4^+$ memory T cell development (Chen and Jensen, 2008).

However, the primary role of B cells lies in provision of antigen-specific humoral immunity, and although they contribute to the orchestration of T cell responses, their significance in priming of T cells is limited. In viral infections and tumour challenges, cellular adaptive immune responses are required, and these are provided by T lymphocytes.

1.3.2 T lymphocytes

Naïve T lymphocytes bear specific TCR which allow them to recognize peptides presented to them in complexes with MHC molecules on the surface of APC. Upon encounter of a T cell with its cognate peptide-MHC complex, in a process referred to as T cell priming, the T cell differentiates and expands into effector cells.

For successful differentiation and expansion of naïve T lymphocytes, the interaction between TCR and MHC-peptide complex, typically referred to as signal 1 of T cell priming, requires the exchange of further signals between T cell and APC. Signal 2 is conveyed by cell surface markers, such as, for example, CD28 expressed by T cells

and so-called co-stimulatory molecules CD80 and CD86, found on APC. A third signal is conveyed by soluble factors, present in the form of cytokines secreted by both APC, T cell and other cells of the immediate environment.

In order to convey signals 2 and 3, DC require activation as provided by PAMP on invading pathogens. In absence of DC activation, signal 1 alone is exchanged between DC and T cell, which is thought to promote naïve T cell inactivation by anergy or deletion, leading to peripheral tolerance (Reis e Sousa, 2006).

T cells expressing the surface marker CD8 recognize antigen peptides presented to them on MHC class I molecules. They have the ability to rapidly induce apoptosis of a target cell by release of perforins and granzymes, and due to these cytotoxic properties, CTL are of paramount importance in viral infections (Young et al., 1990). Degranulation and release of cytotoxic agents resembles the mechanism of action of the neutrophils of the innate immune system. However, the killing induced by CTL is highly specific and only affects the target cells that display the relevant peptide on MHC class I molecules, leaving uninfected bystander cells unharmed. This is achieved by exclusive release of cytotoxic agents precisely at the point of contact between the infected cell and CTL rather than their undirected release into the cell's surroundings such as seen in neutrophils (Podack et al., 1988; Berke, 1994).

For efficient cross-priming of naïve CD8⁺ T cells by DC, adequate signals 2 and 3 have to be in place to accompany TCR interaction with MHC-peptide complex. DC activation leading to expression of high levels of co-stimulatory molecules CD80, CD86 and secretion of cytokines is induced by interaction of DC surface molecule CD40 and CD40 ligand (CD40L) expressed on T_H cells (Bennett et al., 1997; Bennett et al., 1998), but can equally be triggered by PRR stimulation (Reis e Sousa, 2004). Interestingly, certain stimuli such as dsRNA recognized by TLR3 are particularly efficient at promoting cross-priming of CTL by DC (Schulz et al., 2005), and this is in part due to induction of type 1 interferon which potentiates this process (Le Bon et al., 2006).

It is evident that by stimulation of specific PRR, pathogens elicit distinct activation patterns of innate immune cells including DC. By differential secretion of cytokines in response to varying stimuli, DC transmit information about the nature of the pathogen to T cells and shape the adaptive immune response. Characteristics of signal 3 determine to a large extent the polarization of naïve CD4⁺ T cells, which, in contrast to naïve CD8⁺ T cells, have the ability to differentiate into diverse effector cell types with contrasting functions (Diebold, 2008).

The most prominent and well understood amongst T_H cells are T_H1 and T_H2 cells. T_H1 responses are typically induced in presence of the cytokine IL-12. T_H1 cells activate macrophages that have engulfed pathogen material, enhancing the macrophages' microbicidal activity. Further, by production of the cytokines IFN γ , TNF α and IL-2, T_H1 cells enhance priming of CTL, boosting cellular immunity (Liu et al., 2013).

In contrast, T_H2 cells primarily promote immunoglobulin class switching in B lymphocytes and are therefore crucial to the orchestration of the humoral immune response, important for clearing of infections with extracellular pathogens (Diebold, 2008; Liu et al., 2013). While DC have the potential to deliver distinct signal 3 depending on the activating stimulus, tissue derived factors such as TSLP-1 (Pedroza-Gonzalez et al., 2011) and mediators secreted by other leukocyte populations can also affect the polarization of T cell priming. For instance, T_H2 cells are induced in presence of the cytokine IL-4 secreted by surrounding T cells and activated mast cells, basophils and eosinophils of the innate immune system (Luzina et al., 2012). T_H2 cells typically produce IL-4, IL-5 and IL-13, self-perpetuating their own induction.

The polarization of T_H cell responses towards one effector cell type or the other is increasingly understood to be a dynamic process, with different T_H cell populations present during an immune response, mutually influencing their own induction. For instance, promotion of T_H1 and T_H2 responses may be in part regulated by a further class of T_H cells that has been identified in the last decade, and is referred to as T_H17 cells due to substantial production of the cytokine IL-17 (Harrington et al., 2005; Park

et al., 2005). This subset is induced during T cell priming in presence of IL-6, IL-23 and TGF β as signal 3 cytokines. T_H17 cells enhance neutrophil activation, and they therefore indirectly amplify the immune response mechanisms directed at extracellular pathogens. It has recently emerged that T_H17 cells can also be harmful to the host by perpetuating chronic inflammation, for example in rheumatologic conditions and autoimmunity (Gullick et al., 2013). In contrast, CD4⁺ regulatory T cells (T reg), characterized by the expression of transcription factor FoxP3, play an important role in prevention of autoimmunity, with numerous studies documenting the detrimental consequences of T reg paucity or malfunction (Ochs et al., 2005; Liu et al., 2013). T reg are induced in presence of the immunosuppressive cytokine TGF β , and their primary function is the regulation of the immune response by contact-dependent inhibition of APC and T cells and production of immunosuppressive cytokines including IL-10 and TGF β (von Boehmer and Daniel, 2013). T reg induction can also be a result of naïve T cell priming by an unactivated DC that can not convey signals 2 and 3 (Reis e Sousa, 2006).

Collectively, the induction of CTL and T_H cell responses by DC is dependent on the DC activation status. Lack of activation by PAMP in sterile inflammation can result in T cell anergy and antigen tolerance, which in the case of cancer is detrimental. Delivery of PRR stimuli to DC in form of TLR agonists for instigation of a tumour specific adaptive immune response is a much applied strategy in cancer immunotherapy. In particular, induction of cellular immunity in form of CTL and T_H1 responses is highly desirable. Activation of DC subsets, notably those able to cross-present TAA, with appropriate TLR agonists, such as synthetic compounds that mimic viral infection, holds promise for future concepts in cancer immunotherapy.

1.4 Cancer and the immune system

The oldest descriptions of cancer in humans are found in the ancient Egyptian document ‘Edwin Smith Papyrus’, dating back to the year 3000 BC. Described as a lump in breast tissue, the author concluded that there was no treatment for this disease. Several other reports originating from different ancient cultures referring to tumour-like growths have been discovered, and in the 4th century BC, the Greek physician Hippocrates finally coined the term ‘carcinoma’. In Greek, this word refers to a crab, most likely applied to the disease because the finger-like projections of the tumours called to mind the body shape of a crab. The Roman physician Celsus later translated the Greek term into ‘cancer’, the Latin word for crab, and respective translations are widely used in many languages to date (Hajdu, 2011).

Today, cancer is the second leading cause of death in high-income countries after cardiovascular diseases (WHO, 2011). Malignant neoplasms can develop from practically any cell in the human body. Fundamentally, cancer is a disease of tissue growth regulation failure, leading to uncontrolled division of cells. Mutations in genes of a cell that promote or inhibit its growth and proliferation can occur due to many different causes, ranging from environmental factors such as radiation, exposure to toxins in form of alcohol or tobacco to infections, hormones and hereditary genetic predispositions. It is very challenging to assign individual risk factors their relative contributions to the development of cancer, and multiple factors are most likely necessary. This notion is supported by the fact that identical exposure to a toxin can lead to malignant neoplasm in one individual, while another remains unharmed. Similarly, not all patients carrying germline mutations of selected genes predisposing them to development of carcinoma will be affected in their lifetime. This suggests that several ‘check points’ have to be overcome by a cancer cell to thrive. The immune system plays a considerable role in this regard, with increasing evidence pointing towards its importance in tumour surveillance and immunoediting.

1.4.1 Cancer immunoediting

The concept that adaptive immunity is responsible for preventing cancer development in immunocompetent hosts was first postulated by Burnet and Thomas in the late 1950s. Early clinical observations of increased incidence of cancer in patients receiving immunosuppressive therapy following organ transplantation supported this idea, and the proof for this and a better understanding of the underlying mechanisms was gradually obtained through the use of animal models. Today, it is widely recognized that the immune system can prevent cancer formation, but it is also evident that even in immunocompetent hosts, it often fails to do so. This basic concept is summarized by the cancer immunoediting hypothesis, a theory that postulates that from an immunological point of view, tumour formation can be described in three sequential phases termed ‘elimination’, ‘equilibration’ and ‘escape’ (Schreiber et al., 2011).

The elimination phase describes a state where the innate and adaptive immune systems work together to detect the presence of a developing tumour and destroy it before it becomes clinically apparent. A detailed understanding of how the immune system is alerted to the presence of malignant cells is yet to be elucidated, but potential mechanisms include release of damage-associated molecular patterns (DAMP) such as high mobility group box 1 (HMGB1) released from dying tumour cells or stress ligands including MICA/B that are frequently expressed on tumour cells (Guerra et al., 2008). It is challenging to truly prove and observe the elimination phase *in vivo*, but its existence is implied by greater susceptibility to neoplasia in immunodeficient mice as compared to wildtype animals (Vesely et al., 2011). Similarly, in humans, despite sophisticated and balanced immunosuppressive regimens used in transplantation nowadays, immunocompromised patients remain more prone to development of malignancy (Gutierrez-Dalmau and Campistol, 2007).

Tumour cell variants that survive the stage of elimination enter the phase of equilibrium. Here, the immune system maintains residual tumour cells in a functional state of dormancy. These cells have the potential to resume growth at a later stage, possibly even after decades. Strong evidence for the existence of the immunological equilibrium phase of cancer immunoediting was provided by a study where immunocompetent mice that harboured occult tumour cells after carcinogen exposure remained tumour free for long periods of time, but developed tumours after ablation of T lymphocytes (Koebel et al., 2007). Equilibrium is thought to be the longest phase which can possibly extend throughout the lifetime of the host. However, tumour cells that have acquired immunoevasive mutations can 'escape' the adaptive immune processes of the equilibrium phase and proceed to grow into manifest tumours.

Progression from equilibrium to escape can occur due to alterations of the tumour cells which lead to reduced immune recognition, such as loss of TAA, or loss of MHC class I proteins that present TAA. Equally, intracellular antigen processing in tumour cells can be impaired, for instance through loss of transporter proteins associated with antigen processing (TAP) which are crucial to shuttling of antigen to the endoplasmatic reticulum (ER) and hence an important step towards loading of TAA onto MHC class I molecules (Dunn et al., 2002; Khong and Restifo, 2002). Further mechanisms of tumour escape include increased resistance to the cytotoxic effects of immunity, for example through induction of anti-apoptotic mechanisms (Vesely et al., 2011). However, escape can equally result from the establishment of immunosuppression in the tumour microenvironment (Radoja et al., 2000). Through production of soluble mediators such as vascular endothelial growth factor α (VEGF α), transforming growth factor β (TGF β), IL-10 and indoleamine 2,3-dioxygenase (IDO) tumours can promote an immunosuppressive state. Further, recruitment and induction of T reg and myeloid-derived suppressor cells (MDSC) can inhibit host-protective antitumour immune responses (Schreiber et al., 2011). With the understanding of cancer immunoediting, it becomes apparent that those tumour cells

that have survived to grow into clinically established tumours are those variants that have developed and continue to exploit successful strategies to circumvent the natural tumour-suppressive mechanisms of immunity. An immunosuppressive environment therefore often persists in established malignant tumours, which can be detrimental to patients' prognosis and survival. Clinical studies have demonstrated that presence of T reg, MDSC and immunosuppressive cytokines are correlated with poor disease outcomes (Sato et al., 2005; Mustea et al., 2006; Hart et al., 2011). Some tumours are more prone to promote immunosuppression than others, with ovarian carcinoma being a prominent example of a tumour which is particularly efficient at fostering an immunosuppressive environment (Yigit et al., 2010).

1.4.2 Ovarian carcinoma

Several studies suggest an association between chronic pelvic inflammatory disease, an inflammatory condition of the female genital tract, and development of ovarian carcinoma (Kisielewski et al., 2013). Chronic inflammation is accompanied by genotoxic stress and enhanced cellular proliferation, angiogenesis and tissue invasion (Grivennikov et al., 2010). Notably, a chronic inflammatory environment leads to immunosuppression, an important mechanism of tumour escape (Baniyash, 2006). The existence of an immunosuppressive environment in established ovarian malignancies is well documented (Yigit et al., 2010) and is increasingly thought to also contribute to early tumour formation.

However, the aetiology of ovarian carcinoma is multifactorial and comprises a number of non-immunological risk factors including hormone replacement therapy, obesity, nulliparity, and personal history of other malignancies, most notably breast cancer. Several genetic mutations that pre-dispose women to development of ovarian carcinoma have been identified and include mutations in breast cancer genes

(BRCA)-1 and -2. As their name suggests, germline mutations in the BRCA genes were originally identified as susceptibility factors for breast cancer (Hall et al., 1990), but carriers bear a considerable risk of developing other types of cancer as well such as pancreatic cancer, and most importantly ovarian carcinoma. The lifetime risk of a female bearing a germline mutation in BRCA-1 or BRCA-2 to develop ovarian carcinoma is approximately 30%, although such incidence estimations do vary considerably between different reports (Nicoletto et al., 2001).

Several types of ovarian carcinoma have been identified based on their histological and morphological properties. Their classification is rather complex and has been subject to changes over the last decade (Gurung et al., 2013). Five subtypes of ovarian carcinoma can be distinguished according to their molecular and pathological features, including clear cell, mucinous, endometrioid, low-grade serous and high-grade serous carcinoma (HGSC). The latter represents the most frequent tumour type constituting over 70% of cases (Gurung et al., 2013), and our study was conducted with material exclusively from patients suffering from HGSC. This tumour type was for a long time believed to arise from the ovarian surface epithelium, but more recent reports suggest that the cells of origin may, in fact, be fimbrial and therefore attributed to the fallopian tube (Delair and Soslow, 2012). Pathological features of HGSC include marked nuclear atypia and very high mitotic index. Genetic mutations of the tumour suppressor protein p53 are characteristic of this disease and are found in 97% of cases. Further molecular attributes of HGSC include BRCA loss and alterations in signalling pathways including forkhead box protein M1 (FoxM1), NOTCH and retinoblastoma protein (Gurung et al., 2013).

A major factor responsible for poor prognosis and a low survival rate of HGSC patients is the lack of early symptoms, leading to the vast majority of patients being diagnosed at an advanced stage of the disease. A hallmark symptom of ovarian carcinoma is the development of ascites, and although this occurs at rather late stages, it is frequently the first sign that alarms the patient. Formation of ascites is a condition

during which liquid accumulates in the peritoneal cavity as a result of an imbalance between fluid production and resorption. The pathophysiology of ascites is multifactorial; the obstruction of venous and lymphatic vessels caused by tumour cells, reduced intravascular protein, increased capillary permeability as well as hormonal changes influencing the renal excretion all contribute to the development of ascites (Chung and Kozuch, 2008). The accumulated volume can often reach several litres and can lead to abdominal swelling, abdominal pain, nausea and vomiting. In order to relieve patients of these symptoms, multidisciplinary therapeutic concepts have been developed. An important part of symptomatic therapy is paracentesis, a procedure during which the liquid is drained out of the peritoneal cavity through a canule. This ensures rapid improvement of the patient's condition but is purely symptomatic and therefore often not lasting, requiring repetition (Chung and Kozuch, 2008).

Ascites harbours tumour cells as well as a considerable infiltration of leukocytes and a multitude of cytokines, chemokines and growth factors (Giuntoli et al., 2009a). T reg, MDSC and M2 macrophages all form part of the cellular composition of malignant ovarian tumours, as do soluble immunosuppressive proteins including VEGF α , IL-10 and TGF β (Conejo-Garcia et al., 2004; Sato et al., 2005; Yigit et al., 2010; Hart et al., 2011; Yigit et al., 2011a). Clinical studies attribute a tumour-promoting role to these factors. Equally, the importance of pro-inflammatory cytokines in advancement and dissemination of established ovarian tumours is convincingly documented (Kulbe et al., 2007), once more highlighting a link between immunosuppression and chronic inflammation in malignant tumours and the significance of such an environment on disease progression. Interestingly, some reports suggest that the immune composition of tumours may impede the efficacy of chemotherapeutic agents. The cytokine IL-6 is implied to reduce the responsiveness of ovarian tumours to paclitaxel and cisplatin, which constitute the first line of chemotherapy currently used for treatment of ovarian carcinoma (Wang et al., 2010; Maccio and Madeddu, 2012).

Relapse due to chemoresistance is indeed a major cause of concern in ovarian carcinoma and an important reason for poor long-term survival.

While chemotherapy represents an important component of current treatment regimes, cytoreductive de-bulking surgery remains the first line of therapy in ovarian carcinoma. The successful removal of tumour mass is highly desirable and considered the most important prognostic factor. However, optimal de-bulking is very complicated to achieve due to the typical peritoneal dissemination of ovarian carcinoma. Large numbers of small tumour nodules are distributed throughout the peritoneal cavity, and their complete excision is frequently not possible. Hence, despite the fact that even advanced tumours including the late stage IIIc of the disease as classified by Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) remain confined to the peritoneal cavity, the current therapeutic measures are not adequate to conquer the challenges of this disease, and novel approaches are paramount for improvement of ovarian carcinoma patient survival.

Due to an abundance of leukocyte subsets present in the tumour environment, immunotherapeutic interventions represent an appealing experimental approach to novel curative strategies. Cytotoxic CD8⁺ T cells can be detected in ascites and in solid ovarian tumours, and a high CTL/Treg ratio was found to be prognostically favourable (Sato et al., 2005;Giuntoli et al., 2009a). DC are also present in tumour tissue and ascites of ovarian carcinoma patients (Wertel et al., 2008), and although in murine models of the disease they have been attributed a pro-tumorigenic role, possibly by inducing tumour vascularisation (Conejo-Garcia et al., 2004;Huarte et al., 2008) and suppressing T cell function, some evidence suggests that T cell stimulatory capacity of DC could be induced by TLR-mediated activation (Scarlett et al., 2009). However, soluble factors of the tumour environment can affect DC function (Gabrilovich et al., 1996;Conejo-Garcia et al., 2004), and future vaccination schemes aiming to harness DC subsets in situ therefore must consider immunosuppressive factors within the tumour environment and their impact on DC functionality. Targeting

and activation of selected DC subsets with TLR agonists *in vivo* for cancer immunotherapy constitutes an important line of research in our laboratory, and in this study, we therefore investigated how soluble factors in ovarian carcinoma associated ascites may influence TLR-mediated DC activation.

1.4.3 Cancer immunotherapy

Frequent failure of established cancer therapies including surgery, chemotherapy and radiation perpetuate the search for new therapeutic strategies, with immunotherapy gaining increasing recognition and importance in the field of oncology. Most immunotherapeutic concepts have only just reached the stage of pre- or early clinical trials despite the fact that the potential of our immune system to eliminate malignant tumours has been known to scientists for centuries. Reports dating back to years BC describe regression of malignancies in patients suffering from coincidental infections, and some clinicians made early curative attempts at cancer by applying local septic dressings to tumours, inducing tissue inflammation and high fever with the hope of thereby activating the immune system to eliminate the malignant mass. William B. Coley, an American surgeon, is considered a pioneer of the modern age in the field of cancer immunotherapy. Unlike his predecessors, Coley recognized the danger of sepsis imposed by the administration of live bacteria to tumour tissue. Early in his career, he therefore introduced what later became known as “Coley’s toxins” – a vaccine containing two species of killed bacteria, *Streptococcus pyogenes* and *Serratia marcescens*. Coley hypothesized that such killed pathogens would still be able to elicit an immune response without posing the same risks as live, replicating strains of bacteria. Coley’s protocols were widely used by other physicians of the era and although failure and severe side effects were not sparse, many stories of success were reported (Hoption Cann et al., 2003).

From today's understanding, Coley's approach was successful due to the presence of PAMP on bacteria and other pathogens. Coley's vaccine consisted of a cocktail of killed bacteria strains, which contained PAMP in the form of cell wall components and nucleic acids from the bacterial genome which lead to APC activation by triggering of PRR.

Decades later, this basic concept is still relevant in cancer immunotherapy. The ultimate goal of any immunotherapeutic approach is the priming and expansion of tumour-specific CD8⁺ cytotoxic T cells and their elimination of malignant cells. As professional APC with the ability to cross-present TAA, DC are pivotal to successful CTL priming. However, their ability to instigate an anti-tumour immune response is dependent on their activation.

Cellular therapies which rely on *ex vivo* generated and expanded DC frequently employ defined cocktails of cytokines for DC activation. In these experimental approaches, DC are usually generated from monocytes by culture with GM-CSF and IL-4 to obtain monocyte-derived DC. Alternatively, preparations of PBMC are used. Before re-injection into to the patient's circulation, the APC are loaded with TAA and cultured with specific cytokines to ensure their activation and subsequent tumour-specific T cell priming *in vivo* (Harada, 2010). A prominent example of such personalized DC vaccination is the therapy with Sipuleucel-T (Provenge®), approved in 2010 by the United States Food and Drug Administration (FDA) for treatment of prostate cancer. The outcome of several clinical trials with Provenge® led to disappointing results (Huber et al., 2012). Cellular DC vaccinations are nevertheless considered promising by many researchers and clinical trials are ongoing. However, their personalized nature and therefore technically demanding preparation and high cost are regarded as limiting by others.

An alternative and much promising approach is targeting of DC for *in vivo* antigen delivery by use of antibodies specific for surface receptors expressed on selected DC subsets, such as DEC-205 (Hawiger et al., 2001; Bonifaz et al., 2002), DNGR-1

(CLEC9A) (Sancho et al., 2008) or many others. Biochemically cross-linked conjugates or fusion proteins of antibody and antigen are used for vaccination, but in initial studies, it has been shown that unless DC are additionally activated by an adjuvant, vaccination results in induction of peripheral antigen tolerance (Bonifaz et al., 2002). Use of cytokines as done for *ex vivo* generated monocyte-derived DC is not ideal for *in vivo* activation of primary DC subsets, because the half-life of many cytokines in serum is short and their systemic administration further poses considerable risk of toxicity and undesired side effects. Moreover, the ability of cytokines to activate DC *in vivo* is debatable, with increasing evidence suggesting that direct activation via PRR is required to obtain DC with the ability to prime effective T cell responses (Joffre et al., 2009).

All DC subsets express several TLR, and TLR agonists therefore represent suitable adjuvants frequently used in vaccination for DC activation *in vivo* (Duthie et al., 2011). Some studies rely on systemic administration of soluble TLR agonists in conjunction with the targeting antibody-antigen complex (Trumpfheller et al., 2008), but in more recent and sophisticated approaches, conjugation of TLR agonists to the antibody-antigen complexes is attempted (Kreutz et al., 2012). Such targeted delivery of the TLR-agonist together with antigen ensures on one hand that DC which present the relevant antigen are activated and can prime antigen-specific CTL, and prevents side effects of systemic TLR agonist application on the other hand.

To date, experience with DC vaccines in ovarian carcinoma is very limited. Several small trials using *ex vivo* generated DC were conducted but have led to rather disappointing results (Stiff et al., 2013). Studies combining chemotherapy with vaccination with TAA-loaded DC were well tolerated, elicited anti-tumour immune responses in some patients (Kandalaft et al., 2013), and prolonged survival (Chu et al., 2012). However, the cohorts in all these studies to date were small and the results therefore do not allow conclusive interpretations. Nevertheless, immunotherapy in ovarian carcinoma remains a promising field of research, with identification of several

ovarian carcinoma associated TAA including Her2/neu, NY-ESO-1 and MUC1 paving the way for future DC based peptide vaccination concepts (Thibodeaux and Curiel, 2011). A complication that persists and may impede immunotherapeutic strategies is the immunosuppressive environment of ovarian carcinoma fostered by the tumour (Yigit et al., 2010). Expression and interaction of inhibitory molecules on DC and T cells are parameters associated with immune suppression in the ovarian carcinoma environment (Kryczek et al., 2006; Krempski et al., 2011). Blockade of co-inhibitory pathways, so-called ‘checkpoint-blockade’, is an attractive clinical direction to boost anticancer immunity in patients, and this concept is being exploited by use of selective antibodies for cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1) and others (Pardoll, 2012). In addition, soluble immunosuppressive factors which are abundant in the ovarian carcinoma environment and in ascites (Yigit et al., 2011a; Yigit et al., 2011b) may equally hinder the efficacy of anti-tumour vaccinations. Due to its confinement to the peritoneal compartment, an understanding of the local immune environment can inform and improve future immunotherapeutic strategies in this disease considerably by identifying important aspects that constitute hurdles on the way towards instigation of efficacious anti-tumour immune responses.

1.5 Aims of this study

In this study, we aim to elucidate the influence of soluble factors in ascites obtained from patients suffering from high-grade serous ovarian carcinoma on activation of human DC subsets by TLR agonists *in vitro*. In particular, we are interested to investigate

- (1) if TLR-mediated DC activation is influenced by ovarian carcinoma associated ascites, and whether influences differ upon use of different TLR agonists.
- (2) which factors in ovarian carcinoma associated ascites are responsible for observed effects on TLR-mediated DC activation and whether the influence exerted by these factors can be overcome.
- (3) whether distinct human DC subsets are affected differently by ovarian carcinoma associated ascites upon TLR-mediated activation.

Our long-term aim is to contribute to a better understanding of the tumour environment in ovarian carcinoma and to identify strategies that promote the efficacy of immunotherapeutic interventions in patients with malignant disease.

Chapter 2

Material & Methods

2.1 General laboratory equipment

Gilson ‘Pipetman’ single channel pipettes P2, P10, P20, P100, P200, P1000

(Gilson, Luton, UK)

Rainin ‘Pipet-Lite’ multi channel pipettes L50, L300 (Anachem, Luton, UK)

Rainin disposable pipette tips for all pipette sizes (Anachem)

Gilson ‘Pipetting Aid’ (Gilson)

Stripette disposables 5ml, 10ml, 25ml (Costar® Sigma-Aldrich, Dorset, UK)

Nunc-Immuno ‘Wash 12’ ELISA washer (Nunc, Roskilde, Denmark) **with attached vacuum pump** (Fisher Scientific, Loughborough, UK)

Microcentrifuge ‘Centrifuge 5424’ (Eppendorf, Stevenage, UK)

Centrifuge ‘5810R’ (Eppendorf)

CO2 incubator (Sanyo Panasonic, Berkshire, UK)

Tissue culture hood (Labcaire Scientific Laboratory Supplies, Hessle, UK)

Laboratory refrigerator (4°C) / freezer (-20°C) (Vestfrost, Esbjerg, Denmark)

Revco laboratory freezer (-86°C) (Thermo Scientific, Cramlington, UK)

Inverted microscope (Motic, Wetzlar, Germany)

Upright microscope (Ceti Medline, Oxon, UK)

Vortex ‘Genie2’ (Scientific Industries, Bohemia, New York, U.S.A.)

Titramax 100 Microplate shaker (Heidolph, Saffron Walden, UK)

2.2 Tissue culture reagents

RPMI 1640 medium (Invitrogen, Paisley, UK)

Fetal calf serum (FCS) (Sigma-Aldrich, Dorset, UK)

Glutamine (Invitrogen)

Penicillin/Streptomycin (Invitrogen)

β -Mercaptoethanol (2-ME) (Gibco Life Technologies, Paisley, UK)

1x Phosphate-buffered saline (PBS) (Gibco Life Technologies)

Ethylene-diamine-tetra-acetic acid (EDTA) (Sigma-Aldrich)

2.3 Patient specimens

Material:

Centrifuge tubes, 50 ml (Corning Inc., Amsterdam, The Netherlands)

Centrifuge tubes, 15ml (VWR, Lutterworth, UK)

FCS (Sigma-Aldrich)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)

CryoTube® Vials, 1.8 ml (Thermo Scientific)

Syringes, 10 ml (Beckton Dickinson (BD), Oxford, UK)

sterile filters, 0.2 μ m pore size (Appleton Woods, Birmingham, UK)

Sample collection and storage protocol:

The collection of ascites for this study was approved by the Guy's Research Ethics Committee (REC Number: 09/H0804/45). Informed consent was obtained from all patients.

Ascites from patients suffering from epithelial ovarian carcinoma was collected by paracentesis (n = 9; Dr. Raju, Department of Gynaecological Oncology, St Thomas' Hospital, London, UK). Peritoneal fluid from patients with benign ovarian conditions (fibroadenoma n = 1, fibrothecoma n = 2, benign cyst n = 2 or cystadenoma n = 1) was collected during surgery (Dr. Raju, Department of Gynaecological Oncology, St. Thomas Hospital, London, and Dr. Ahmed, University of Oxford, Nuffield Department of Obstetrics and Gynecology, Oxford, UK). Characteristics of collected specimens are summarized in Table 2.1.

Upon receipt, fluid samples were divided into centrifuge tubes and centrifuged (1200 rpm, 10 minutes), allowing the separation of the fluid into a cellular fraction and non-cellular supernatant. The cells were frozen in FCS with 10% DMSO and stored in CryoTube® vials at -196°C in liquid nitrogen tanks. The non-cellular supernatant was stored in aliquots in 15 ml centrifuge tubes at -80°C. Upon thawing, before use in cell cultures, the non-cellular fluid was passed through a syringe-driven sterile filter.

<u>Patient code</u>	<u>Tumour type</u>	<u>FIGO Stage</u>	<u>Therapy at the time of ascites collection</u>
Malignant			
OC4	HGSC	IV	before de-bulking
OC5	HGSC	IV	before de-bulking
OC6	HGSC	IIIc	before de-bulking
OC9	HGSC	unknown	before de-bulking
OC13	HGSC	unknown	before de-bulking
OC23	HGSC	IIIB	before de-bulking
OC25	HGSC	IIIB	before de-bulking, post-chemotherapy
OC26	ovarian adenocarcinoma	unknown	before de-bulking
Benign			
OC14	mucinous cystadenoma	N/A	perioperative
OC24	fibroma cystadenoma	N/A	perioperative
OC27	thecoma	N/A	perioperative
OC34	fibrothecoma	N/A	perioperative
OC35	benign cyst	N/A	perioperative
OC36	benign fimbrial cyst	N/A	perioperative

Table 2.1 Characteristics of patient specimens recruited for this study

This table provides a summary of all patient specimens used in this study. For data protection reasons, patient samples were anonymized and numbered ('patient code'). Indicated is the histological tumour type, FIGO stage, and therapy received. All patients with malignant tumours received surgical therapy (de-bulking), and ascites samples were obtained by paracentesis before de-bulking surgery. Except for patient OC25, no information about chemotherapy status is available. Surgical removal constituted the only therapy for patients suffering from benign ovarian tumours. Collection of benign peritoneal fluid was carried out during surgery ('perioperative').

2.4 PBMC isolation

Material:

Vacutainer® blood collection tubes, glass, heparinized, 9 ml (BD)

Vacutainer® Push Button Blood Collection Set, 21G (BD)

Alcotip Swabs (Universal Hospital Supplies, Enfield, UK)

‘Buffy Coat’ leukocyte preparations (National Blood Service Tooting, London, UK)

Centrifuge tubes, 50 ml (Corning Inc.)

1 x PBS (Gibco Life Technologies)

Lymphocyte Separation Medium 1077 (PAA Laboratories, Yeovil Somerset, UK)

Trypan blue solution 0.4% (Sigma-Aldrich)

Neubauer counting chamber (Fisher Scientific)

Protocol:

The collection of peripheral blood for this study was approved by the Guy’s Research Ethics Committee (REC Number: 09/H0804/45). Informed consent was obtained from all participants who gave blood by venipuncture.

Peripheral blood from healthy volunteers was obtained by venipuncture and collected in heparinized vacutainer tubes. Alternatively, buffy coat leukocyte preparations were obtained from the National Blood Service. After dilution (1:2 dilution for peripheral blood obtained by venipuncture, 1:4 dilution for buffy coats) with PBS, the blood was layered over Lymphocyte Separation Medium 1077 in 50 ml centrifuge tubes. After centrifugation (1600 rpm, 20 minutes, brake off) the PBMC fraction was harvested into fresh 50 ml centrifuge tubes, pooled and washed twice with PBS (1200 rpm, 10 minutes). An aliquot of cells was added to trypan blue solution 0.4%, which had been diluted 1:5 in PBS, and live cells were counted in an upright microscope at 40x magnification in a Neubauer counting chamber.

2.5 Enrichment of CD14⁺ monocytes

Material:

EasySep[®] Human Monocyte Enrichment Kit (StemCell Technologies, Grenoble, France)

Enrichment medium (1 x PBS containing 2% FCS and 1mM EDTA)

Polystyrene tubes, round-bottom, 14 ml (BD)

EasySep[®] “The Big Easy” Silver Magnet (StemCell Technologies)

Protocol:

CD14⁺ monocytes were isolated from PBMCs by negative selection using the EasySep[®] Human Monocyte Enrichment Kit following the manufacturer's instructions. Briefly, PBMC were re-suspended in recommended enrichment medium in a 14 ml polystyrene round-bottom tube at a density of 5×10^7 cells/ml. The antibody enrichment cocktail provided in the kit was added at a concentration of 50 μ l/ml cell suspension, followed by an incubation at 4°C for 10 minutes. Magnetic bead particles were added at a concentration of 50 μ l/ml cells and after a further incubation for 5 minutes at 4°C, the tube was placed in an EasySep[®] magnet at room temperature (RT) for 2.5 minutes to allow magnetic separation of cells. After this time, the magnet with the tube was inverted and the fraction containing untouched CD14⁺ monocytes was collected in a fresh tube. The remaining magnetically labeled unwanted cells were retained inside the original tube and discarded. The purity of the enriched CD14⁺ monocytes was assessed by flow cytometry and was typically over 95%.

2.6 Generation of monocyte-derived DC

Material:

Complete R10 medium (RPMI 1640 supplemented with 10% FCS, 2mM glutamine, 100U/ml penicillin, 100mg/ml streptomycin, 50 μ M 2-ME)

Granulocyte-macrophage colony stimulating factor (GM-CSF) (R&D Systems, Abingdon, UK)

Interleukin-4 (IL-4) (R&D Systems)

6-well tissue culture plates (Costar® Sigma-Aldrich)

PBS w/EDTA (1x PBS supplemented with 5mM EDTA)

Protocol:

Enriched CD14⁺ monocytes were seeded on 6-well-plates at a density of 6-9x10⁶ cells/well in 3 ml complete R10 medium and 20 ng/ml GM-CSF and 20 ng/ml IL-4. Cells were cultured at 37°C in a 5% CO₂ atmosphere for 7 days. GM-CSF and IL-4 were replenished on day 2 and day 5 along with 500 μ l fresh complete R10 medium per well. Cells were harvested on day 7 of culture with PBS w/EDTA. The differentiation of monocytes into monocyte-derived DC was verified by flow cytometric analysis of the surface markers CD14 and CD1a.

2.7 Activation assay of monocyte-derived DC

Material:

Complete R10 medium

96-well tissue culture plates, flat-bottom (Costar® Sigma-Aldrich)

PBS w/EDTA (1x PBS supplemented with 5mM EDTA)

TLR agonists:

- R848 (stock conc. 2 mg/ml) (Invivogen, Toulouse, France)
- LPS (stock conc. 5 mg/ml) (Invivogen)
- Poly I:C (stock conc. 2 mg/ml) (GE Healthcare, Amersham, UK)

Neutralizing antibodies:

- α IL-10 antibody (mouse IgG2b clone 25209) (R&D Systems)
- α TGF- β antibody (mouse IgG1 clone 27235) (R&D Systems)
- α VEGF α antibody (mouse IgG2b clone 26503) (R&D Systems)
- α LIF antibody (mouse IgG2b clone 9824) (R&D Systems)
- α IL-6 antibody (mouse IgG2b clone 1936) (R&D Systems)
- α PGE₂ antibody (mouse IgG1 clone 2B5) (Cayman Europe, Tallinn, Estonia)
- mouse IgG1 isotype control antibody (clone 11711) (R&D Systems)
- mouse IgG2b isotype control antibody (clone 20116) (R&D Systems)

Patient specimens:

- malignant ascites from ovarian carcinoma patients
- peritoneal fluid from patients with benign ovarian tumours

Protocol:

Monocyte-derived DC were seeded on 96-well tissue culture plates at a density of 1×10^5 cells/well in complete R10 medium, with a final well volume of 200 μ l. Triplicate wells were used for all samples. TLR agonist concentrations were titrated in pilot experiments (chapter 3, Figure 3.3). Concentrations that induced robust up-

regulation of activation markers were determined as R848 3 µg/ml, LPS 1 µg/ml and poly I:C 100 µg/ml, and these concentrations were used in subsequent experiments throughout the project. Where indicated, sterile filtered, non-cellular peritoneal fluid from patients with malignant or benign ovarian tumours was added to samples to reach either 10% or 25% cell culture concentration. Cells were incubated for 18h overnight at 37°C in a 5% CO₂ atmosphere. Supernatants were harvested and analyzed for cytokine levels. In addition, cells were harvested with PBS containing 5mM EDTA for analysis of surface marker expression by flow cytometry.

In order to identify the factors that suppress DC activation, cells were cultured as described above, with the addition of neutralizing antibodies. The antibodies were added to cell cultures concomitantly with respective TLR agonists. Individual antibodies were used either separately or in combinations with a final concentration of 5 µg/ml per antibody. Respective isotype control antibodies were included in every experiment and were used at equivalent concentrations of 5 µg/ml per antibody.

2.8 Enrichment of primary DC subsets (panDC)

Material:

EasySep[®] Human panDC Enrichment Kit (StemCell Technologies)

Enrichment medium (1 x PBS containing 2% FCS and 1mM EDTA)

Polystyrene tubes, round-bottom, 14 ml (BD)

EasySep[®] “The Big Easy” Silver Magnet (Stemcell Technologies)

Protocol:

Primary DC subsets were isolated from PBMCs by negative selection using EasySep[®] Human panDC Enrichment Kits following the manufacturer’s instructions. Briefly, PBMC were re-suspended in enrichment medium in a 14 ml polystyrene round-bottom tube at a density of 5×10^7 cells/ml. Reagents provided in the respective EasySep[®] negative enrichment kits were added and incubation times were followed according to the manufacturer’s protocols. Subsequently, the tube was placed in an EasySep[®] magnet for a designated amount of time to allow magnetic separation of cells. The magnet with the tube was inverted and the negatively selected fraction containing the desired untouched DC was collected in a fresh centrifuge tube. The magnetically labeled unwanted cells remained bound inside the original tube and were discarded. mDC1 population was further purified by FACS sorting.

2.9 FACS sorting of mDC1 from panDC

Material:

Microcentrifuge vials 1.5ml (Costar® Sigma-Aldrich)

FACS buffer (1 x PBS containing 5mM EDTA, 1% heat-inactivated FCS and 0.02% NaN₃)

CD1c (BDCA-1)-APC conjugated antibody (mouse IgG2a, clone AD5-8E7) (Miltenyi Biotech)

CD19-FITC conjugated antibody (mouse IgG1, clone LT19) (Immunotools)

FACS tubes (BD)

FACS Aria cell sorter (BD)

Protocol:

After enrichment by negative magnetic selection as described above, panDC were transferred into microcentrifuge vials and washed with FACS buffer before incubation with antibodies diluted in FACS buffer (1:10 dilution for CD1c-APC antibody; 1:100 dilution for CD19-FITC antibody) for 30 mins at 4°C in the dark. Cells were then washed 2x and samples were transferred into FACS tubes for cell sorting, which was performed by qualified staff at the cell sorting facility of the Division of Immunology, Infection and Inflammatory Disease. The desired cell fraction, defined as CD1c⁺CD19⁻ cells, was collected in fresh FACS tubes and used further in activation assays.

2.10 Activation assay of primary mDC1

Material:

Complete R10 medium

96-well tissue culture plates, flat-bottom (Costar® Sigma-Aldrich)

PBS w/EDTA (1x PBS supplemented with 5mM EDTA)

TLR agonists:

R848 (stock conc. 2 mg/ml) (Invivogen)

α IL-10 neutralizing antibody (mouse IgG2b clone 25209) (R&D Systems)

Protocol:

mDC1 obtained by FACS sorting as described above were seeded on 96-well tissue culture plates at a density of approximately 6×10^4 cells per well. Triplicate wells were used for all samples. The same concentration of TLR7 agonist R848 as with monocyte-derived DC was used (3 μ g/ml). Where indicated, sterile filtered, non-cellular peritoneal fluid from ovarian carcinoma patients was added to samples to reach 10% final cell culture concentration. Where indicated, α IL-10 neutralizing antibody (5 μ g/ml) was added to samples concomitantly with R848 and ascites. Cells were incubated for 18h overnight at 37°C in a 5% CO₂ atmosphere. Supernatants were harvested and analyzed for cytokine levels. In addition, cells were harvested with PBS containing 5 mM EDTA for analysis of surface marker expression by flow cytometry.

2.11 Depletion of IL-10 or PGE₂ from ascites

2.11.1 Depletion of IL-10 using Protein G coated agarose beads

Material:

Centrifuge tubes, 15ml (VWR)

α IL-10 antibody (mouse IgG2b clone 25209) (R&D Systems)

mouse IgG2b isotype control antibody (clone 20116) (R&D Systems)

Protein G coated agarose beads (Thermo Scientific)

Protocol:

IL-10 neutralizing antibody or relevant IgG2b isotype control antibody were added to sterile filtered ascites at a concentration of 5 μ g/ml or 20 μ g/ml and the samples were incubated for 30 minutes at 4°C. After addition of 100 μ l Protein G agarose beads the samples were incubated for 30 minutes at room temperature. Samples were centrifuged at 1500 RPM for 5 minutes, and the supernatant was carefully harvested. Centrifugation at identical speed and duration was repeated to ensure removal of residual agarose beads, and carefully harvested ascites supernatant was used in activation assays.

2.11.2 Depletion of IL-10 using Streptavidin magnetic beads

Material:

Centrifuge tubes, 15ml (VWR)

α IL-10 antibody (mouse IgG2b clone 25209) (R&D Systems)

mouse IgG2b isotype control antibody (clone 20116) (R&D Systems)

anti-mouse IgG antibody, biotinylated (goat Ig clone Poly4053) (BioLegend)

Magnetic Streptavidin MicroBeads (Miltenyi Biotec)

LD magnetic columns (Miltenyi Biotec)

MidiMACS magnet and magnetic stand (Miltenyi Biotec)

1 x PBS (Gibco)

Protocol:

α IL-10 neutralizing antibody or respective mouse IgG2b isotype control antibody were added to 3 ml sterile filtered ascites at a concentration of 10 μ g/ml and incubated for 30 minutes at 4°C. Subsequently, biotinylated α -mouse IgG antibody was added to the samples at a concentration of 20 μ g/ml, followed by incubation for 30 minutes at 4°C. Next, Streptavidin MicroBeads were added at a concentration of 100 μ l bead stock solution per 1 ml ascites (300 μ l per sample) and samples were incubated for 15 minutes at 4°C. LD magnetic columns were placed into the block magnet and calibrated with 500 μ l PBS. Each ascites sample was loaded onto a calibrated column, and the flow-through was collected in a fresh centrifuge tube. This procedure was repeated two more times with fresh, PBS calibrated magnetic columns for a total of three passages of ascites samples over columns.

2.11.3 Depletion of IL-10 using Protein G coated magnetic beads

Material:

PureProteome Protein G Magnetic Beads (Millipore, Livingston, UK)

α IL-10 antibody (mouse IgG2b clone 25209) (R&D Systems)

mouse IgG2b isotype control antibody (clone 20116) (R&D Systems)

chromatographically purified mouse IgG (Invitrogen)

PureProteome Magnetic Stand (Millipore)

Microcentrifuge vials 1.5ml (Costar® Sigma-Aldrich)

1 x PBS w/0.1% Tween 20

Protocol:

The manufacturer's (Millipore) protocol for binding of antibodies to Protein G Magnetic Beads was followed. Briefly, 50 μ l Protein G Magnetic Beads were incubated in a microcentrifuge vial with 10 μ g/ml α IL-10 neutralizing antibody or respective mouse IgG2b isotype control antibody for 10 minutes at RT with continuous mixing in the mixing block at 1000 RPM. Subsequently, beads were washed with 500 μ l PBS w/Tween 20 with the help of the magnetic stand. Subsequently, 200 μ g chromatographically purified mouse IgG2b was added to each sample in order to saturate vacant IgG binding sites on Protein G Magnetic Beads. An incubation for 10 minutes at RT in the mixing block at 1000 RPM was followed by 3x washing with 500 μ l PBS w/Tween 20 with help of the magnetic stand. Antibody-coated Protein G Magnetic Beads were added to 1 ml sterile filtered ascites and incubated for 1 hour or overnight at 4°C. After incubation, magnetic beads were removed by placing the microcentrifuge tubes into the magnetic stand and transfer of the bead-free ascites into a fresh microcentrifuge tube. This procedure was repeated two more times to ensure complete removal of magnetic beads.

2.11.4 Depletion of IL-10 or PGE₂ using NHS coated magnetic beads

Material:

PureProteome *N*-Hydroxysuccinimide (NHS) FlexiBind Magnetic Beads (Millipore)

Equilibration Buffer (1mM HCl) (Millipore)

Wash/Coupling Buffer (PBS, pH 7.4) (Millipore)

Quench Buffer (100mM Tris-HCl, 150 mM NaCl, pH 8.0) (Millipore)

Amicon® Ultra-0.5 30K Centrifugal Filter Devices (Millipore)

αIL-10 antibody (mouse IgG2b clone 25209) (R&D Systems)

mouse IgG2b isotype control antibody (clone 20116) (R&D Systems)

αPGE₂ antibody (mouse IgG1 clone 2B5) (Cayman Europe)

mouse IgG1 isotype control antibody (clone 11711) (R&D Systems)

PureProteome Magnetic Stand (Millipore)

Microcentrifuge vials 1.5ml (Costar® Sigma-Aldrich)

Protocol:

To ensure successful coupling of antibody to the PureProteome NHS Magnetic Beads, the antibodies must be present at a concentration of 2 mg/ml or above. The stock concentration of both isotype control antibodies as well as the αIL-10 antibody was 500 µg/ml and a further concentration was therefore required to reach 2 mg/ml. For this, a defined amount of antibody (100µg) in solution was pipetted into an Amicon® Ultra-0.5 30K Device, and centrifuged at 14,000 x g for 7 minutes. The obtained concentrated antibody solution was recovered from the device and the volume was adjusted to 50 µl, resulting in a final antibody concentration of 2 mg/ml. The anti-prostaglandin E₂ (αPGE₂) antibody is supplied at a concentration of 2 mg/ml and no further concentration was therefore required.

For the coupling of antibodies to PureProteome NHS Magnetic Beads, the protocol recommended by the manufacturer's protocol was followed closely. Briefly, beads

were washed 1x with ice-cold Equilibration Buffer and 50 μ l of antibody solution (2 mg/ml) was immediately added, followed by an incubation for 2 hours at RT. Subsequently, beads were washed 5x with Quench Buffer followed by an incubation of 2 hours at RT in Quench Buffer. The beads were washed 4x with Wash/Coupling Buffer. Such antibody-coated beads were then incubated with ascites from ovarian carcinoma patients to deplete the desired cytokines IL-10 or PGE₂. For the depletion, the α IL-10 or α PGE₂ antibody-coated beads and were incubated with ascites overnight at 4°C. To control for the specificity of the depletion, isotype control mouse IgG coated beads were also incubated with ascites samples for mock depletion. After overnight incubation, beads were removed from ascites samples using the PureProteome Magnetic Stand. IL-10 and PGE₂ levels were measured in the depleted samples to verify whether complete and specific removal of the respective proteins was achieved. The depleted ascites was further assessed by mouse IgG-specific ELISA to ensure no unbound residual neutralizing antibody was present in these samples after depletion. Ascites depleted of IL-10, PGE₂ and mock-depleted samples were stored at -80°C until their use in activation assays.

2.12 Enrichment of naïve T cells

Material:

EasySep[®] Human Naïve CD4⁺ T cell Enrichment Kit (StemCell Technologies)

Enrichment medium (1 x PBS containing 2% FCS and 1mM EDTA)

Polystyrene tubes, round-bottom, 14 ml (BD)

EasySep[®] “The Big Easy” Silver Magnet (StemCell Technologies)

Protocol:

Naïve CD4⁺ T cells were isolated from PBMC by negative selection using the EasySep[®] Human Naïve T cell Enrichment Kits following the manufacturer's instructions. Briefly, PBMC were re-suspended in recommended enrichment medium in a 14 ml polystyrene round-bottom tube at a density of 5×10^7 cells/ml. Reagents provided in the respective EasySep[®] negative enrichment kits were added and incubation times were followed according to the manufacturer's protocols. The tube was then placed in an EasySep[®] magnet to allow magnetic separation of cells. After this time, the magnet holding the tube was inverted and the fraction containing untouched naïve T cells was collected in a fresh 14 ml polystyrene round-bottom tube. The remaining magnetically labeled unwanted cells were retained inside the original tube, which was discarded. The tube containing the desired cells was again placed into the EasySep[®] magnet for a further round of separation after which the contents were inverted into a fresh centrifuge tube. The purity of the enriched naïve T cells as assessed by flow cytometry was typically over 98%.

2.13 Allogeneic mixed leukocyte reaction

Material:

1 x PBS (Gibco Life Technologies)

Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Paisley, UK)

FCS (Sigma-Aldrich)

Centrifuge tubes, 15ml (VWR)

Complete R10 medium

96-well tissue culture plates, round-bottom (Costar® Sigma-Aldrich)

Protocol:

Naïve T cells were enriched by negative selection as described above and subsequently labeled with CFSE. For this, T cells were washed twice with PBS and re-suspended in 1 μ M CFSE solution in a 15ml centrifuge tube at a density of 1.0×10^7 cells/ml and incubated at RT for 10 minutes. To quench the staining, a volume of FCS equivalent to that of CFSE solution used was added for 2 minutes at RT, before cells were washed with complete R10 medium and processed further.

Monocyte-derived DC were pre-cultured overnight under varying conditions with or without TLR agonists and/or ascites as described previously. To ensure no carryover of TLR agonists and/or ascites, DC were washed twice with fresh R10 medium before being added to the CFSE-labeled CD4⁺ T cells at ratios of 1:100 or 1:200 (DC : T cells). Samples were performed in triplicates in round-bottom 96-well plates, with 50 000 naïve T cells per well. The cultures were incubated at 37°C in a 5% CO₂ atmosphere for 6 days. After harvest, cells were analyzed by flow cytometry to assess T cell proliferation by CFSE dilution and T cell polarization by intracellular cytokine staining.

2.14 Flow Cytometry

Material:

Microcentrifuge vials 1.5ml (Costar® Sigma-Aldrich)

FACS buffer (1 x PBS containing 5mM EDTA, 1% heat-inactivated FCS and 0.02% NaN₃)

Fixation Buffer (BioLegend, Cambridge, UK)

Permeabilization Buffer (BioLegend)

Monensin Solution (BioLegend)

FoxP3 Staining Buffer Set (eBioscience, Hatfield, UK)

LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen)

Paraformaldehyde 37% (VWR)

FACS tubes (BD)

FACS Canto II analyzer

FACS Clean solution (BD)

FACS Rinse solution (BD)

FACS Flow solution (BD)

FlowJo Software (Treestar. Ashland, Oregon, U.S.A.)

Specificity	Fluorochromes	Isotype	Clone	Staining dilution	Manufacturer
<u>Surface markers:</u>					
CD1a	PE	mouse IgG1κ	HI149	1:5	BD
CD1c (BDCA-1)	APC	mouse IgG2a	AD5-8E7	1:10	Miltenyi
CD3	APC-Cy7	mouse IgG1κ	SK7	1:20	BD
CD4	APC	mouse IgG1	MEM-241	1:100	Immunotools
CD8	APC	mouse IgG2a	MEM-31	1:100	Immunotools
CD14	FITC	mouse IgG1	MEM-18	1:100	Immunotools
HLA-DR	PerCP-Cy5.5	mouse IgG2a	L243	1:5	BD
CD40	PE	mouse IgG2a	LOB7/6	1:10	AbD Serotec
CD45RA	PE	mouse IgG2b	MEM-56	1:100	Immunotools
CD86	V450	mouse IgG1κ	2331 (FUN-1)	1:10	BD
CD123	APC	mouse IgG2a	AC145	1:10	Miltenyi
CD141 (BDCA-3)	APC	mouse IgG1	AD5-14H12	1:10	Miltenyi
CD273	PE	mouse IgG1κ	MIH18	1:10	BD
CD274	PE-Cy7	mouse IgG1κ	MIH1	1:10	BD
CD303 (BDCA-2)	FITC	mouse IgG1	AC144	1:10	Miltenyi
<u>Intracellular markers:</u>					
FoxP3	AlexaFluor 647	mouse IgG1	259D/C7	1:10	BD
IFN-γ	PerCP-Cy5.5	mouse IgG1κ	4S.B3	1:10	eBioscience
IL-10	BrillViolet 421	rat IgG1κ	JES3-9D7	1:10	BioLegend
IL-17A	PE	mouse IgG1	BL168	1:10	BioLegend

Table 2.2 Compilation of antibodies used for flow cytometry

This table provides a summary of all antibodies used throughout this study for flow cytometry analysis. Specificity, isotype, clone and host species are listed together with dilutions used for staining.

Protocol:

All antibodies used in this study are listed in Table 2.1 together with their working dilutions.

Samples for surface marker staining were transferred into microcentrifuge vials and washed with FACS buffer before incubation with antibodies diluted in FACS buffer for 30 mins at 4°C in the dark. Cells were then washed 2x and samples were transferred into FACS tubes for acquisition.

For intracellular detection of cytokines, cell were incubated with Monensin solution for 6 hours before harvesting. Surface marker staining was performed as described above, after which cells were fixed by incubation with Fixation Buffer for 20 mins in the dark at RT. After centrifugation, Fixation Buffer was discarded and cells were washed 5x in Permeabilization Buffer. Subsequently, cells were stained for 20 mins in the dark at RT with desired antibodies diluted in Permeabilization Buffer. Cells were washed 2x with Permeabilization Buffer and 1x in FACS buffer before being transferred into FACS tubes for acquisition.

For FoxP3 staining, T cells were first stained with a CD3 antibody according to the surface marker staining procedure described above. After the second wash with FACS buffer, the samples were pulse vortexed and incubated with FoxP3 Fixation/Permeabilization working solution for 30 mins in the dark at RT. After permeabilization, cells were washed 1x with Permeabilization Buffer, and subsequently incubated with the FoxP3 antibody diluted in Permeabilization Buffer for 30 mins in the dark at RT. After washing 1x with Permeabilization Buffer and 1x with FACS buffer, cells were transferred into FACS tubes for acquisition.

Viability assessment was performed for selected experiments to investigate whether ovarian carcinoma associated ascites influenced viability of monocyte-derived DC. This staining was therefore conducted separately and not in combination with other surface markers. The protocol recommended by the manufacturer was followed. Briefly, fluorescent reactive dye was reconstituted in DMSO provided in the kit and

diluted 1:1000 in PBS. Cells were incubated with this working solution for 30 minutes on ice and washed once. Subsequently, cells were fixed in paraformaldehyde (4% working dilution in PBS) for 15 minutes at RT. Cells were washed once with FACS buffer and transferred into FACS tubes for acquisition.

2.15 Cytokine analysis of cell culture supernatants

2.15.1 Flow Cytomix

Material:

Flow Cytomix Simplex Kits specific for detection of human IL-6, TNF- α , IL-1 β , IL-2, IL-4, IL-10, IFN- γ , IL-17A/F (eBioscience)

Flow Cytomix Basic Kit (eBioscience)

FACS tubes (BD)

FACS Canto II (BD)

Flow Cytomix Analysis Software (eBioscience)

Protocol:

For analysis of cytokines in cell culture supernatants obtained from activation assays with monocyte-derived DC, a combination of Flow Cytomix Simplex Kits for detection of IL-6, TNF- α , IL-10 and IL-1 β was used. For IL-6 and TNF α , supernatants were diluted 1:10 with assay buffer provided in the Flow Cytomix basic kit in order to ensure the values fell within sensitivity range of the assay. For measurement of IL-10 and IL-1 β , supernatants were used undiluted. For analysis of cell culture supernatants from allogeneic MLR experiments, Flow Cytomix Simplex Kits for detection of IL-2, IL-4, IL-10, IFN- γ and IL-17A/F were used in combination. The manufacturer's protocol for Simplex Kits was followed using dilution buffers, reagents and a 96-well filter plate all provided in the Flow Cytomix Basic Kit. Samples were transferred into FACS tubes for acquisition on the FACS Canto II, and data was analyzed and quantified using Flow Cytomix Analysis Software.

2.15.2 ELISA for detection of TNF- α

Material:

Human TNF- α ELISA MAX Standard Set (BioLegend; cat. no. 430203) containing

- TNF- α capture antibody, purified (isotype and clone not specified by BioLegend)
- TNF- α detection antibody, biotinylated (isotype and clone not specified by BioLegend)
- TNF- α standard, lyophilized
- Avidin-HRP

Immuno MAXISORP plates, 96 wells (Nunc, Roskilde, Denmark)

Wash Buffer (1 x PBS containing 0.05% Tween 20)

1 x PBS (Gibco Life Technologies)

Assay Diluent (1 x PBS containing 10% heat-inactivated FCS)

3,3',5,5'-Tetramethylbenzidine (TMB) Substrate (BD)

Stop Solution (2NH₂SO₄)

Protocol:

ELISA plates were coated with TNF- α capture antibody diluted in PBS (1:200 dilution of stock solution; absolute antibody concentration is not specified by BioLegend) and incubated overnight at 4°C. All subsequent incubations were performed on a microplate shaker, and between each two steps, plates were washed 4x with ELISA wash buffer. Non-specific binding sites were blocked with Assay Diluent and incubated for 1 hour at RT, after which TNF- α standard and samples were added to triplicate wells appropriately diluted in Assay Diluent. Samples without TLR agonists or those stimulated with TLR3 agonist poly I:C were measured neat. Samples stimulated with R848 or LPS were diluted 1:10 or 1:50 in order to ensure that the

values fell within the sensitivity range of the assay. Where α IL-10 antibody had been added to cultures, a dilution of supernatants of 1:100 or 1:200 was necessary. After incubation for 2 hours at RT, TNF- α detection antibody diluted in Assay Diluent (1:200 dilution of stock solution; absolute antibody concentration is not specified by BioLegend) was added to all wells for 1 hour at RT followed by application of avidin-HRP for 30 minutes at RT. A thorough wash 5x was followed by application of TMB substrate. The reaction was stopped by adding stop solution, optical density (OD) was measured on an ELISA microplate reader and all samples evaluated with reference to the standard curve for cytokine quantification.

2.15.3 ELISA for detection of IL-6 and IL-12p40

Material:

Immuno MAXISORP plates, 96 wells (Nunc)

Coating Buffer (0.1M Na₂HPO₄, pH 9.0)

Wash Buffer (1 x PBS containing 0.05% Tween 20)

ELISA Buffer (1 x PBS containing 2.5% FCS and 0.02% NaN₃)

IL-6 capture antibody, purified (BD) (rat IgG1 clone MQ2-13A5; 0.5 mg/ml)

IL-6 detection antibody, biotinylated (BD) (rat IgG2a clone MQ2-39C3; 0.5 mg/ml)

IL-6 Standard, in buffered solution (BD)

IL-12p40 capture antibody, purified (BD) (mouse IgG1 clone C8.3; 1 mg/ml)

IL-12p40 detection antibody, biotinylated (BD) (mouse IgG1 clone C8.6; 0.5 mg/ml)

IL-12p40 Standard, in buffered solution (BD)

ExtrAvidin®-Alkaline Phosphatase (Sigma-Aldrich)

SIGMAFAST p-Nitrophenyl phosphate (PNPP) Tablets (Sigma-Aldrich)

Protocol:

ELISA plates were coated with respective capture antibody diluted in coating buffer (for IL-6 1:125 dilution of stock, 4 µg/ml coating concentration; for IL-12p40 1:200 dilution of stock, 5 µg/ml coating concentration) and incubated overnight at 4°C. Plates were washed 2x with wash buffer and non-specific binding sites were blocked by incubating all wells with ELISA buffer for 2 hours at RT. After washing 2x, standard and samples were added to triplicate wells at appropriate dilutions in ELISA buffer. For both IL-6 and IL-12, supernatants were diluted in the same way. Samples without TLR agonists or those stimulated with TLR3 agonist poly I:C were measured neat. Samples stimulated with R848 or LPS were diluted 1:10 or 1:50 in order to ensure that the values fell within the sensitivity range of the assay. Where αIL-10 antibody had been added to cultures, a dilution of supernatants of 1:50 or 1:100 was

necessary. After overnight incubation at 4°C, plates were washed 4x and respective detection antibody (for both IL-6 and IL-12p40 1:200 dilution of stock, 2.5 µg/ml detection antibody concentration) was added to all wells and allowed to incubate for 1 hour at RT. After 4x washing, Extravidin-AP was added to all wells and plates were incubated for 1 hour at RT. A 4x wash was followed by application of PNPP substrate tablets dissolved in water. OD was measured on an ELISA microplate reader and all samples evaluated with reference to the standard curve for cytokine quantification.

2.15.4 ELISA for detection of mouse IgG1 and mouse IgG2b

Material:

Immuno MAXISORP plates, 96 wells (Nunc)

1 x PBS (Gibco Life Technologies)

Wash Buffer (1 x PBS containing 0.05% Tween 20)

Assay Diluent (1 x PBS containing 10% heat-inactivated FCS)

Anti-mouse IgG1 antibody, purified (rat IgG clone RMG1-1) (BioLegend)

Anti-mouse IgG2b antibody, purified (rat IgG clone RMG2b-1) (BioLegend)

Anti-mouse IgG antibody, biotinylated (goat Ig clone Poly4053) (BioLegend)

ExtrAvidin®-Alkaline Phosphatase (Sigma-Aldrich)

SIGMAFAST p-Nitrophenyl phosphate (PNPP) Tablets (Sigma-Aldrich)

Protocol:

ELISA plates were coated with respective capture antibody diluted in PBS (for both anti-mouse IgG1 and IgG2b 1:250 dilution of stock, 2 µg/ml coating concentration) and incubated overnight at 4°C. Plates were washed 2x with wash buffer and non-specific binding sites were blocked by incubating all wells with Assay Diluent for 2 hours at RT. After washing 2x, standard and samples were added to triplicate wells at appropriate dilutions in ELISA buffer. The anticipated IgG concentrations were difficult to estimate, and therefore a wide range of sample dilutions (undiluted, 1:10, 1:100, 1:1000, 1:10000) was used in all ELISA experiments to ensure that the values fell within the sensitivity range of the assay. After overnight incubation at 4°C, plates were washed 4x and biotinylated detection antibody (1:1000 dilution of stock, 0.5 µg/ml detection antibody concentration) was added to all wells and allowed to incubate for 1 hour at RT. After 4x washing, Extravidin-AP was added to all wells and plates were incubated for 1 hour at RT. A 4x wash was followed by application of PNPP substrate tablets dissolved in water. OD was measured on an ELISA microplate

reader and all samples evaluated with reference to the standard curve for cytokine quantification.

2.16 Cytokine analysis of malignant ascites and benign peritoneal fluid samples

Material:

PGE₂ Parameter Assay kit (Arbor Assays, Ann Arbor, MI, U.S.A.)

CTLA-4 Platinum ELISA kit (eBioscience)

Arginase-1 ELISA kit (Hycult Biotech, Uden, The Netherlands)

LIF ELISA kit (R&D)

VEGF- α , CCL18, IL-6, IL-10, TNF α , IFN- γ and IL-17 matched antibody pairs (all R&D)

Protocol:

For measurement of PGE₂, CTLA-4 and Arginase-1, the manufacturer's specific instructions provided with each kit were followed closely. For CTLA-4, a dilution of ascites samples of 1:2 was used. For PGE₂, samples were measured diluted either 1:2, 1:10 or 1:20. For Arginase-1, ascites samples were diluted 1:2 or 1:10.

The measurements of LIF, VEGF- α , CCL18, IL-6, IL-10, TNF α , IFN- γ and IL-17 in malignant ascites and benign peritoneal fluid samples were performed by Dr. Ann Jagger and Dr. Hayley Evans from the research group of Dr. Leonie Taams (Centre for Molecular & Cellular Biology of Inflammation, King's College London).

2.17 Statistical analysis

Data were analyzed using PRISM Graph Software (La Jolla, California, U.S.A.). Appropriate statistical tests were applied for different experiments, taking into consideration the composition of each experiment, relevant experimental cohorts and scientific questions. Differences were considered significant at a p-value < 0.05.

Chapter 3

Impact of ovarian carcinoma associated ascites on TLR-mediated monocyte-derived DC activation

Introduction

Development of ascites is a clinical hallmark of many peritoneal cancers, with all ovarian carcinoma patients at late stages of their disease presenting with substantial amounts of accumulated liquid within the peritoneal cavity. As outlined in chapter 1, the composition of ovarian carcinoma associated ascites is complex and harbours a plethora of soluble immunosuppressive factors (Yigit et al., 2011a). These proteins within the fluid influence and act upon surrounding cells of the tumour environment, including DC which have been shown to be present in ascites from ovarian carcinoma patients (Wertel et al., 2008). Many experimental immunotherapeutic approaches aim to activate DC subsets in situ in various tumour models, and these concepts are being explored in our laboratory as well as by other research groups worldwide (Tacken and Figdor, 2011; Ueno et al., 2011; Kreutz et al., 2012). TLR agonists are frequently used as adjuvants in this context, their immunostimulatory capacity and potential to boost vaccine efficacy having been convincingly documented by numerous studies (Duthie et al., 2011).

One of the challenges faced in vaccination strategies aiming to target and activate DC in situ is that of interference of local immunosuppressive factors with the activatory stimulus delivered by the vaccine. These can be present either in form of soluble components of the tumour environment, which have been shown in previous studies to influence DC phenotype and function in ovarian carcinoma (Gabrilovich et al., 1996; Conejo-Garcia et al., 2004). However, direct contact between DC and other

suppressive leukocytes as well as tumour cells may also alter activation and phenotype of DC. It is of course desirable to gradually gain an understanding of these complex influences of the tumour environment in their entity, but a dissection into cell-cell interactions and soluble factors can be very helpful when trying to attribute observed effects to individual factors. In our study, we therefore removed tumour cells and infiltrating leukocytes by centrifugation and sterile filtration before use of ascites samples in our assays, allowing us to focus exclusively on soluble factors within the ovarian carcinoma environment and their impact on TLR-mediated DC activation, bearing in mind however that *in vivo*, cell-cell interactions equally play a role in immunosuppression in ovarian carcinoma. Understanding the immediate tumour environment is of considerable importance and promise in ovarian carcinoma. Given its localized nature with restriction of metastases to the peritoneal cavity even in late stages of the disease, tackling the hurdles that these local factors pose to immuotherapeutic approaches could improve experimental concepts even in advanced clinical stages.

With particular regard to our study, we were interested to learn whether soluble factors within ovarian carcinoma associated ascites influence activation of DC with TLR agonists and if so, how DC reconcile these signals from the tumour environment with TLR-mediated activation. DC are a rare leukocyte type, only comprising approximately 1% of mononuclear cells in human peripheral blood. In the early years after their characterization (Van Voorhis et al., 1982), the lack of standardized methods for enrichment of DC as well as their paucity made studies of primary human DC very challenging and therefore sparse. While simple methods for purification of DC from PBMC have become widely available, the low numbers of this cell type in peripheral blood continue to pose a challenge and restrict the feasibility and execution of assays performed with primary DC populations. The discovery that DC could be generated from the much more frequent peripheral blood monocytes *in vitro* with the use of recombinant GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994; Bender et al.,

1996;Pickl et al., 1996;Romani et al., 1996) propelled the research on human DC forward immensely, allowing for the generation of large numbers of these cells and making detailed studies of human DC characteristics and functions in many different experimental settings practicable.

In our study, we were interested to investigate TLR-mediated activation of human DC in presence of ovarian carcinoma associated ascites. No protocols for these particular study aims had been previously established in our laboratory, and it was therefore necessary to design and optimize experimental procedures. The ethical approval of our study only allows for withdrawal of 100 ml of peripheral blood from each healthy volunteer at any one time, and the numbers of primary DC that could be obtained from this material would therefore not be sufficient to perform assays with numerous samples. We intended to employ several different TLR agonists and varied culture conditions in terms of ascites concentration, and for these reasons, we have chosen to use monocyte-derived DC as an experimental model to study the influence of ovarian carcinoma associated ascites on TLR-mediated activation of DC. The insights gained from these experiments would then allow us to perform assays with primary DC using a few chosen conditions affording a much smaller number of samples.

Monocyte-derived DC share many properties of primary myeloid DC and are therefore regarded as a suitable model system for *in vitro* studies of DC. They exhibit DC morphology and express phenotypic markers typical for myeloid DC, such as high levels of CD1a, CD11c, HLA-DR, CD40 and CD80, and lack the monocyte marker CD14. Their antigen presentation and T cell stimulatory capacity in MLR is remarkable and exceeds that of other APC such as B cells (Sallusto and Lanzavecchia, 1994;Pickl et al., 1996). Despite these indisputable similarities to primary myeloid DC, caution should be taken when extrapolating findings to primary DC subsets. It remains unclear to what extent DC generation from monocytes takes place *in vivo*. Some reports suggest that monocytes differentiate into DC under inflammatory conditions, and such inflammatory DC, referred to as 'Tip-DC' (tumor-necrosis-factor and

inducible nitric oxide synthase-producing inflammatory DC), could represent an equivalent to *in vitro* generated monocyte-derived DC (Rivollier et al., 2012). However, Tip-DC have been shown to develop normally in GM-CSF receptor deficient mice (Greter et al., 2012). With increasing awareness of the dynamic nature of blood leukocyte populations, studies relying on identification and classification of DC subsets according to their development pathways from bone marrow progenitors are gaining more recognition and importance (Satpathy et al., 2012). In this respect, regardless of what their *in vivo* equivalent may be, monocyte-derived DC are defined as progeny of the monocyte/macrophage lineage, and it is therefore only natural that they bear certain differences to primary DC subsets. It is imperative that these distinctions are considered when interpreting and drawing conclusions from studies using monocyte-derived DC.

Despite these reservations, monocyte-derived DC remain a useful and widely accepted tool in the field of human DC research, and the following chapters outline our findings when using these cells to elucidate the influence that soluble factors within the ovarian carcinoma environment exert on TLR-mediated DC activation.

3.1 *In vitro* generation of monocyte-derived DC

Human primary DC subsets are sparse in peripheral blood, and the amount of blood that can be drawn at any one timepoint from a healthy volunteer for research purposes in our study is restricted to 100 ml due to ethical regulations. Myeloid DC constitute approximately 0.5-1% of PBMC, and hence the number of primary DC obtained from these amounts of peripheral blood is insufficient when performing activation assays comprising several different cell culture conditions. Therefore, for this study, we used *in vitro* generated monocyte-derived DC as a model system to study the influence of ovarian carcinoma-associated ascites on TLR-mediated DC activation.

Monocytes were isolated from peripheral blood of healthy volunteers by negative magnetic selection. The purity of the isolated CD14⁺ monocytes as assessed by flow cytometry was consistently above 90% (Figure 3.1 A). The untouched monocytes were cultured for 7 days in presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml), resulting in their differentiation into monocyte-derived DC over this time period. In the course of their differentiation, the cells underwent morphological changes resulting in increased granularity and cell size as detected by flow cytometry (Figure 3.1 B; please note the different scale in both forward scatter (FSC; x-axis) and sideward scatter (SSC; y-axis)). The differentiation was verified by flow-cytometric surface marker analysis (Figure 3.1 C). Surface marker CD14 is expressed in high levels on monocytes, and is gradually down-regulated in the course of differentiation into monocyte-derived DC, although low levels are detectable even after 7 days. Surface marker CD1a behaves in opposing fashion, with freshly isolated monocytes being negative for this protein while it is highly expressed by monocyte-derived DC. Interestingly, two distinct populations, CD1a^{int} and CD1a^{hi}, can be identified on day 7. A previous report has described this phenomenon and the authors attributed differential cytokine secretion profiles and capacity to skew T_H cell differentiation to these two populations (Chang et al., 2000). In this project, we used both populations

together and did not further investigate their functional differences in our assays. Cells were harvested on day 7 and used in activation assays as described below.

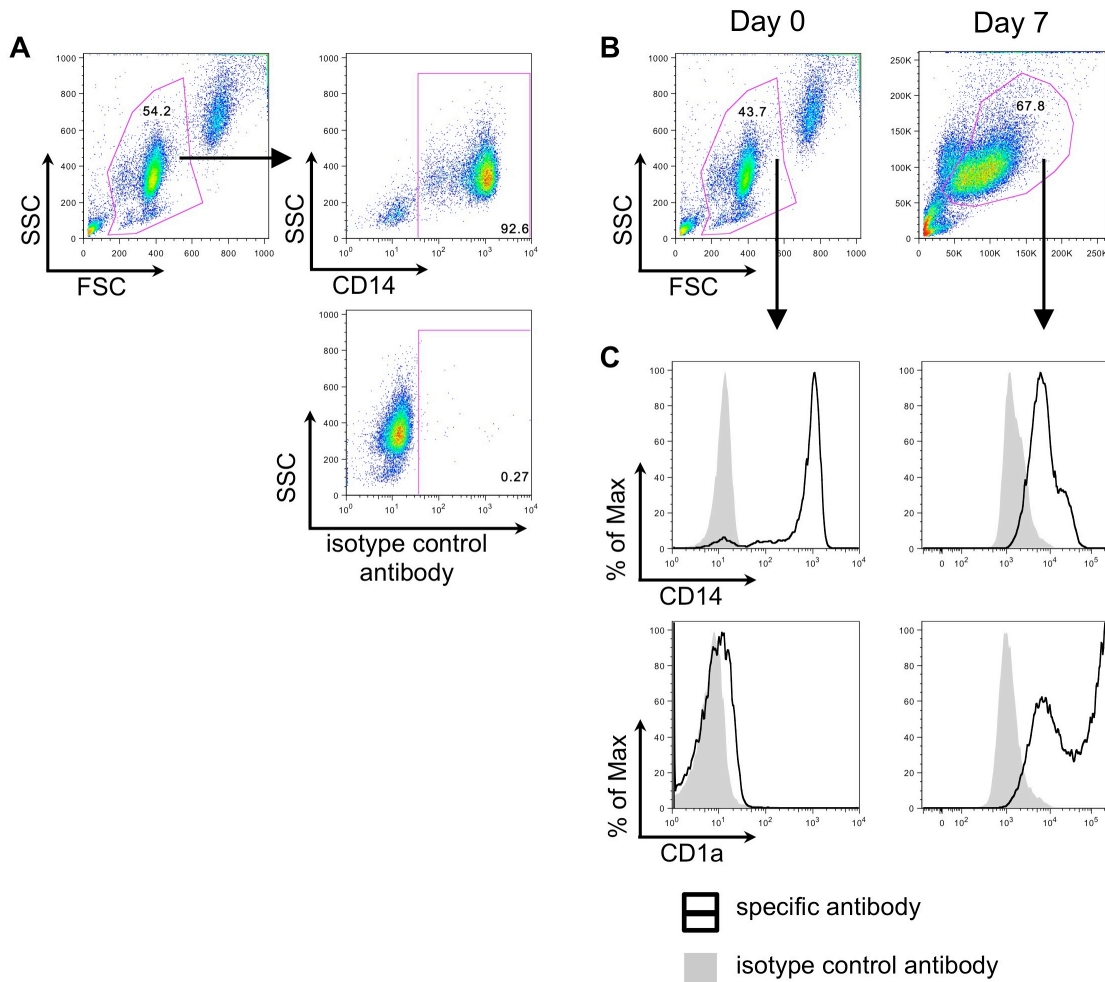


Figure 3.1 Differentiation of CD14⁺ monocytes into monocyte-derived DC

(A) Monocytes from healthy volunteers were enriched from PBMC by negative magnetic selection, and their purity was regularly assessed by flow cytometry. Shown is a representative example of a staining profile, indicating a purity of 92.6% CD14⁺ monocytes after gating on all cells excluding couplets and debris. **(B)** After 7 days of culture in the presence of GM-CSF and IL-4, the size and granularity of the cells increased considerably. Please note the difference in scale on both x- and y-axis. **(C)** The differentiation of monocytes into DC was verified by surface marker staining for CD14 and CD1a. The bigger size of cells on day 7 led to higher autofluorescence and background staining, resulting in higher CD14 and CD1a background levels on day 7 (grey shaded curves).

3.2 Activation of monocyte-derived DC with TLR agonists

In order to study the influence of ovarian carcinoma-associated ascites on TLR-mediated DC activation, the establishment of experimental protocols that ensure a robust activation of monocyte-derived DC with TLR agonists was essential. Monocyte-derived DC have been described to express high levels of TLR2 and TLR4 and low levels of TLR1, TLR3, TLR5, TLR6 and TLR8 (Jarrossay et al., 2001). Based on these reports, we conducted pilot experiments, subjecting monocyte-derived DC to an overnight incubation in complete medium either without stimulant, or with addition of TLR3 agonist poly I:C, TLR4 agonist LPS or TLR7/8 agonist R848 (Figure 3.2). In order to determine the concentrations that induced optimal activation of monocyte-derived DC, three concentrations were chosen for each TLR agonist based on published literature and previous experience in our laboratory, and tested in this initial assay. As a read-out for activation of DC, the cytokine IL-6 was measured by ELISA in cell culture supernatants after overnight incubation (Figure 3.2 A). When cells were cultured in medium only without TLR stimulus, no IL-6 production was detectable (Figure 3.2 A, 'medium' value in the first graph). In response to R848 and LPS, monocyte-derived DC produced substantial levels of IL-6 in a dose-dependent fashion, with higher TLR agonist concentrations showing greater IL-6 induction. The two highest concentrations of TLR3 agonist poly I:C also induced notably higher IL-6 levels than untreated cells, the concentrations reached were however 20- to 40-fold lower than after stimulation with LPS or R848, respectively.

To further verify monocyte-derived DC activation by all three TLR agonists, we assessed the expression of the surface markers CD86, CD40 and HLA-DR by flow cytometry after overnight incubation with the two highest previously tested concentrations of each TLR agonist (Figure 3.2 B). Incubation with R848 and LPS induced a robust up-regulation of all three surface molecules at both tested concentrations. CD86 was also up-regulated in presence of both concentrations of

poly I:C, while HLA-DR up-regulation was only induced by 30µg/ml of poly I:C and CD40 levels remained unchanged in presence of poly I:C altogether.

Our laboratory has extensive experience with CpG oligodeoxynucleotides as potent activators of DC via TLR9, and controversial reports in the literature regarding the expression of TLR9 on monocyte-derived DC (Jarrossay et al., 2001;Hoene et al., 2006;Diebold, 2009) prompted us to include these TLR9 agonists in our pilot assay to assess whether they induce detectable activation of these cells in our experimental setup. Three different classes of CpG oligodeoxynucleotides (CpG-A, CpG-B and CpG-C) were tested at three different concentrations each. We could detect neither an enhanced IL-6 production, nor an increase in CD86 up-regulation compared to untreated cells (data not shown). This lack of activation by CpG oligodeoxynucleotides did not support the previously published reports where TLR9 expression and responsiveness to CpG stimuli were attributed to monocyte-derived DC (Hoene et al., 2006), but were rather in line with studies where lack of response to CpG oligodeoxynucleotides by monocyte-derived DC was seen (Bauer et al., 2001).

Based on the data from these initial assays, for subsequent experiments in this research project, we used TLR3 agonist poly I:C (100 µg/ml), TLR4 agonist LPS (1 µg/ml) and TLR7/8 agonist R848 (3 µg/ml) to study the influence of ovarian carcinoma associated ascites on monocyte-derived DC activation mediated via TLR.

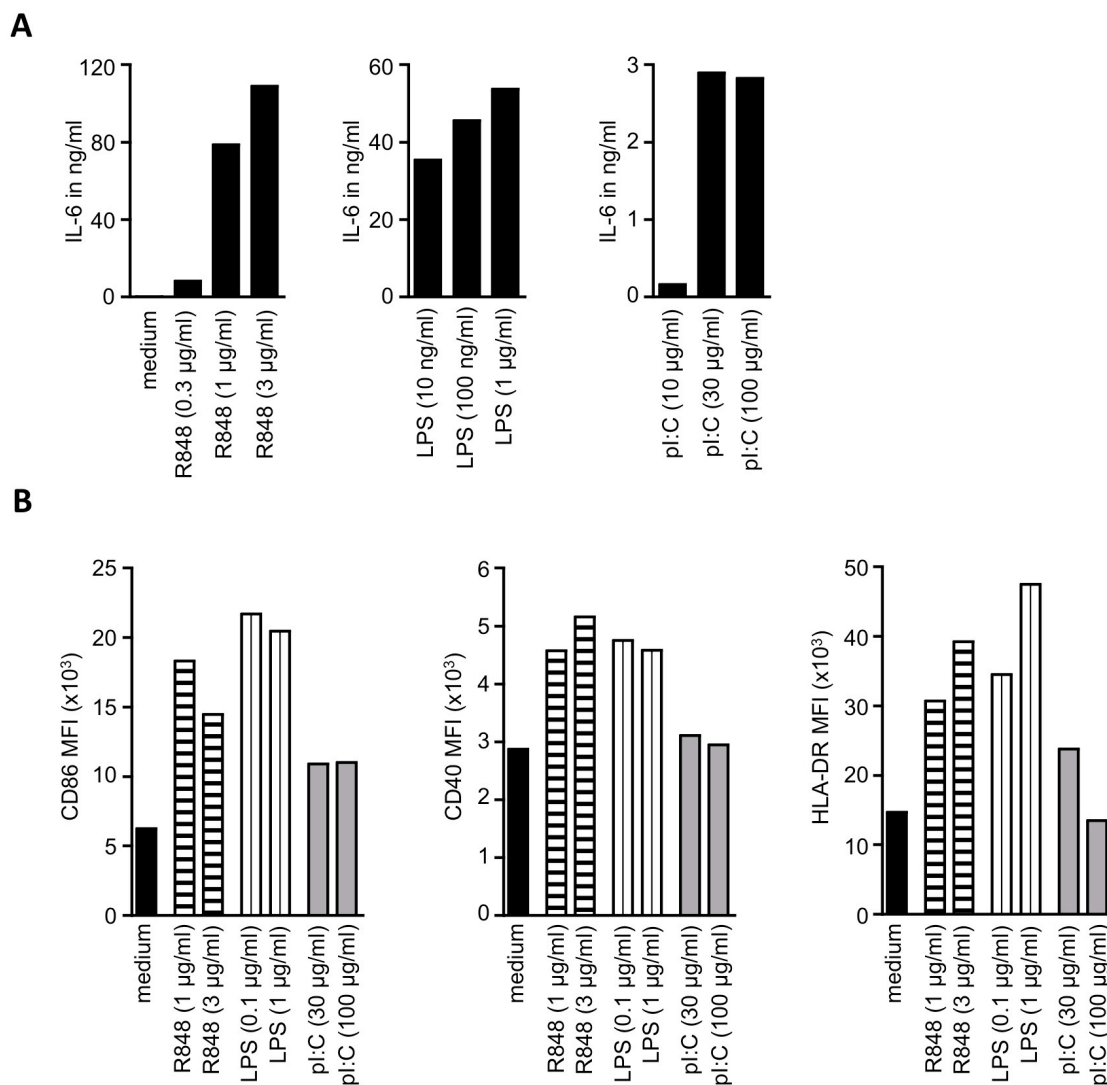


Figure 3.2 Titration of TLR agonists for activation of monocyte-derived DC.

(A) Monocyte-derived DC were cultured overnight in complete medium either in absence of TLR stimuli, or with addition of TLR agonists R848, LPS or poly I:C (pl:C) at the indicated concentrations. IL-6 levels were measured by ELISA in cell culture supernatants, and **(B)** for each TLR agonist, the two concentrations that induced the highest IL-6 levels were further tested with regards to up-regulation of the surface molecules CD86, CD40 and HLA-DR as assessed by flow cytometry. Samples for cytokine assessment were performed in triplicate wells on a 96-well plate, while cells for flow cytometric analysis were cultured in 24-well plates. One pilot experiment was conducted each for cytokine and surface marker assessment (n=1).

3.3 *In vitro* viability of monocyte-derived DC in presence of ovarian carcinoma associated ascites

In order to study the impact of ascites from ovarian carcinoma patients on the activation of monocyte-derived DC in this project, we intended to add ascites to overnight *in vitro* cultures at the concentrations of 10% or 25%. A necessary factor to consider in this experimental setup was the potential influence of ascites on the viability of monocyte-derived DC. This question was important to address, because increased cell death in culture wells containing ascites compared to others could skew our data by influencing the markers that we used as a read-out for DC activation. We therefore performed experiments to assess cell viability after overnight culture in presence of 25% ascites. We studied the influence of ascites on the viability of both cells activated by R848 as well as not activated cells (Figure 3.3). The fixable viability dye used has the ability to react with cell amines, staining amines on the cell surface of live cells resulting in a relatively dim fluorescence (first histogram peak). Dying cells are equally stained on their surface, but in addition, because the integrity of the cell membrane is compromised, the dye penetrates the cells, reacting also with free amines inside the cells. This results in a higher fluorescence signal compared to live cells, detectable as a second peak in the histogram. As a positive control for cell death, we cultured cells in medium not supplemented with FCS (incomplete medium). Here, we detected an increased rate of cell death compared to cells cultured in complete medium, with over 20% versus 5% of cells losing viability after overnight culture. We observed a slight increase in death rate in cells activated with R848 as compared to cells cultured without TLR stimulus (approx. 10% versus 5%). Importantly however, cell death was unaffected by presence of 25% ascites regardless of whether cells were concomitantly activated with R848 or not. These data confirmed that the use of

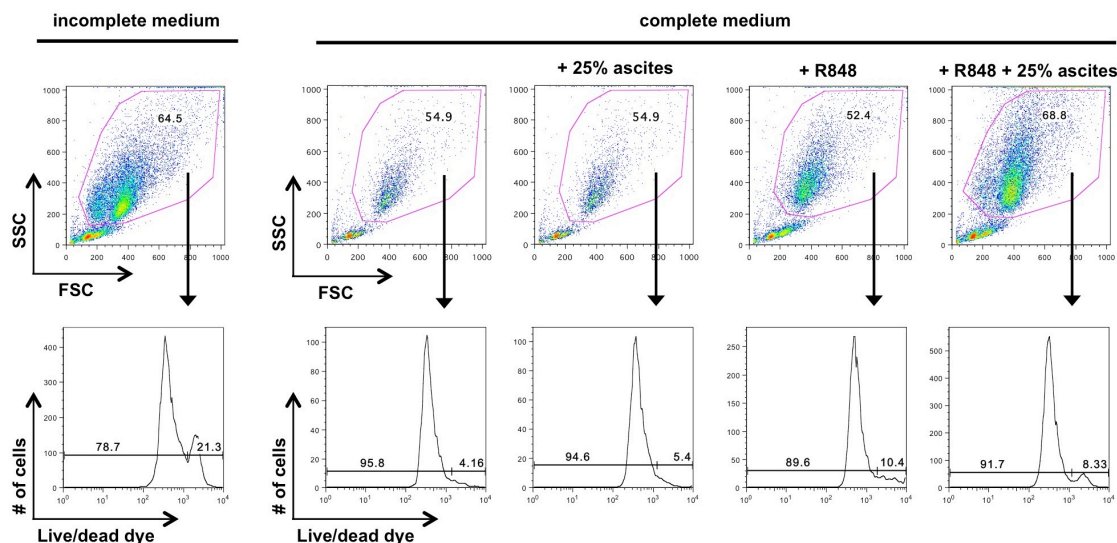


Figure 3.3 Ovarian carcinoma associated ascites does not impair cell viability *in vitro*.

Monocyte-derived DC were cultured overnight in presence or absence of ascites from ovarian carcinoma patients (25% final concentration in cell culture) with or without TLR7/8 agonist R848. After harvest, cells were stained with a fixable viability dye (Live/Dead Aqua®). The upper panel shows gating on all cells excluding debris in the forward vs. sideward scatter. In the lower panel, viable cells are shown as the left histogram peak (lower fluorescence intensity), while dying cells show higher fluorescence levels and are visible as the second histogram peak. A representative example of 3 independent experiments is shown (n=1).

up to 25% of ascites from ovarian carcinoma patients in our *in vitro* cultures of monocyte-derived DC did not compromise cell viability and this experimental setup was therefore suited to study the influence of ascites on TLR-mediated DC activation.

3.4 Ovarian carcinoma associated ascites partially suppresses TLR-mediated monocyte-derived DC activation *in vitro*

Assessment of the effects of ovarian carcinoma associated ascites on the activation of monocyte-derived DC by TLR agonists *in vitro* was a central point of interest in our study. Having established protocols that ensure robust activation of monocyte-derived DC with the TLR agonists poly I:C, LPS and R848 (see above; Figure 3.2), in the following set of experiments, we added cell-free, sterile-filtered ascites to our cultures to study its influence on the cells' activation. To provide sufficient nourishment and growth conditions for overnight cultures of monocyte-derived DC, complete, supplemented medium was used and the concentration of ascites was limited to 10% or 25%.

3.4.1 Ovarian carcinoma associated ascites partially suppresses TLR-mediated up-regulation of CD86 but not CD40, HLA-DR or PD-L1

After overnight incubation under varying cell culture conditions, expression levels of a range of surface markers were measured by flow cytometry. These data were used as a read-out to evaluate monocyte-derived DC activation by TLR agonists *in vitro* in absence versus presence of ovarian carcinoma associated ascites. Upon activation with R848, LPS or poly I:C, we observed a reduced up-regulation of the co-stimulatory molecule CD86 in presence of ascites (Figure 3.4 A). This effect was consistent when treating monocyte-derived DC from the same healthy donor with ascites samples from several ovarian carcinoma patients, as well as when using ascites from one patient in assays with monocyte-derived DC from different healthy controls. We observed statistically significant CD86 suppression at a concentration of 25% ascites in cell culture for all three TLR agonists. When

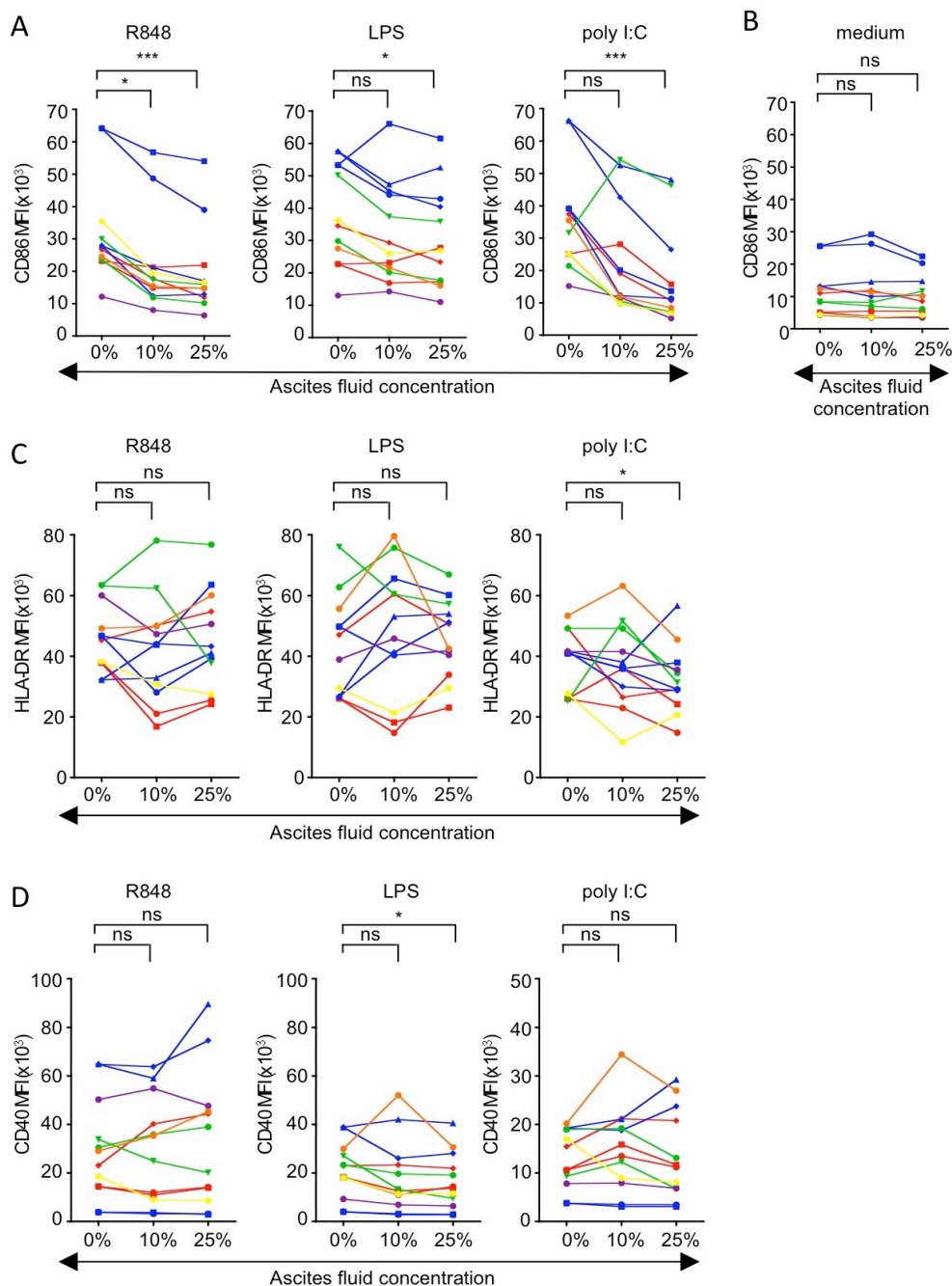


Figure 3.4 Ovarian carcinoma associated ascites partially suppresses TLR-mediated up-regulation of CD86.

(A) Monocyte-derived DC were stimulated overnight with 3 μ g/ml R848, 1 μ g/ml LPS, 100 μ g/ml polyI:C in the presence of 0%, 10% or 25% of ascites from patients with ovarian carcinoma. Alternatively (B) no TLR stimuli were added to cell cultures. The mean fluorescence intensity (MFI) of the surface markers CD86 and (C) HLA-DR and (D) CD40 was assessed by flow cytometry. n=12 (DC from six different healthy volunteers, encoded by six colours), were cultured with ascites from 5 different ovarian carcinoma patients, encoded by different symbols. Altogether, 12 independent experiments were performed.; One-way ANOVA (Friedman test with Dunn post test): * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant.

cells were activated with TLR7/8 agonist R848, the lower concentration of 10% of ascites in culture was already sufficient to significantly reduce CD86 levels. In most experiments, presence of 10% of ascites also led to reduced CD86 levels upon activation with LPS or poly I:C, but this was not statistically significant, most likely due to several donors within the cohorts where no suppression was observed. Cells that were not activated via TLR did not show ascites-induced reduction of CD86 levels (Figure 3.4 B).

The impact of ascites on the up-regulation of surface markers HLA-DR and CD40 (Figure 3.4 C-D) upon TLR activation was not consistent in our experiments and mostly showed no clear suppression pattern. In presence of 25% ovarian carcinoma associated ascites, poly I:C activated DC did show reduced HLA-DR levels, and when activated with LPS, partially impaired CD40 up-regulation was observed. These differences were, however, rather small and somewhat overshadowed by an overall lack of consistency across different blood donors and ascites samples when monitoring these two surface markers.

In a limited number of experiments, we further examined the expression levels of the molecules programmed cell death 1 ligand 1 (PD-L1) (CD274) and OX-40 ligand (OX-40L) (CD252) on monocyte-derived DC, because they are important markers of DC activation and bear essential roles in the process of co-stimulation and/or co-inhibition of T cells during antigen presentation. In these experiments, only 10% of ascites in culture was used in conjunction with TLR activation (Figure 3.5). Upon activation with R848 and LPS, PD-L1 expression was up-regulated but levels were not significantly altered in presence of 10% ovarian carcinoma associated ascites (Figure 3.5 A). Poly I:C did not induce a significant up-regulation of PD-L1, and it is therefore difficult to interpret the reduction of PD-L1 expression in presence of ascites which appears statistically significant. It is important to note that only 3 independent experiments are available and summarized in the graph depicting poly I:C stimulation, and that the MFI values were subject to large variation between donors.

More repeats of this assay using poly I:C would be necessary to conclusively interpret these data.

Assessment of the expression levels of OX-40L indicated that this molecule is not up-regulated on monocyte-derived DC upon stimulation with all three TLR agonists used in our study, and addition of ovarian carcinoma associated ascites equally did not alter OX-40L levels (Figure 3.5 B).

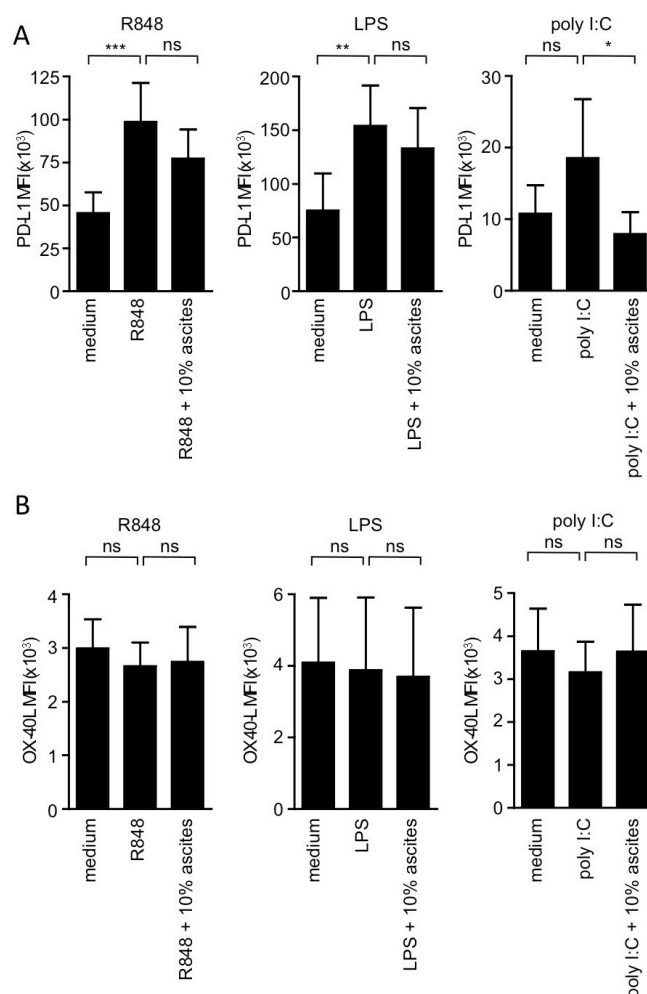


Figure 3.5 Influence of TLR agonists and ovarian carcinoma associated ascites on expression levels of cell surface markers PD-L1 and OX-40L.

Monocyte-derived DC were cultured without stimuli ('medium') or stimulated overnight with 3µg/ml R848, 1µg/ml LPS, 100µg/ml polyI:C. Ovarian carcinoma associated ascites was added to cultures at a final concentration of 10% where indicated. **(A)** The mean fluorescence intensity (MFI) of the surface markers PD-L1 (CD274) and **(B)** OX-40L (CD252) was assessed by flow cytometry. For PD-L1 R848 n=10, LPS n=5, poly I:C n=3. For OX-40L R848 n=6, LPS n=4, poly I:C n=3. One-way ANOVA (Friedman test with Dunn post test): * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant.

3.4.2 Ovarian carcinoma associated ascites partially suppresses TLR-mediated production of IL-6, IL-12p40 and TNF α but not IL-1 β or IL-10

When cultured in complete medium without any stimuli overnight, monocyte-derived DC do not produce detectable levels of any cytokines measured in our study. However, upon activation with TLR agonists, monocyte-derived DC produce an array of cytokines which, in addition to surface marker expression, can function as read-out parameters for cell activation (Figure 3.6 A). In our study, we monitored the levels of the cytokines IL-6, TNF α , IL-12p40, IL-1 β and IL-10, and this provided an additional way to CD86 up-regulation to assess the impact of ascites on TLR-mediated monocyte-derived DC activation in our experimental setup. After overnight cultures, supernatants were harvested and cytokine levels were measured by ELISA.

Upon activation with TLR7/8 agonist R848 and TLR4 agonist LPS, monocyte-derived DC produced high levels of the pro-inflammatory cytokine IL-6, and we further observed high production of TNF α and IL-12p40. In comparison, the TLR3 agonist poly I:C induced low levels of IL-6 and TNF α and failed to induce production of IL-12p40 (Figure 3.6 A). Importantly for our scientific question, in cultures activated with R848 and LPS, in presence of both 10% or 25% ascites, the levels of the cytokines IL-6, IL-12p40 and TNF α were significantly reduced (Figure 3.6 B). The suppression was robust and reproducible with monocyte-driven DC from different healthy donors as well as ascites samples from several ovarian carcinoma patients. Upon poly I:C activation, addition of ascites did not significantly reduce IL-6 production, and although statistical analysis indicated suppressed TNF α levels upon addition of ascites, the importance of this finding may be limited due to the overall low levels of this cytokine induced in response to TLR3 stimulation (Figure 3.6 B).

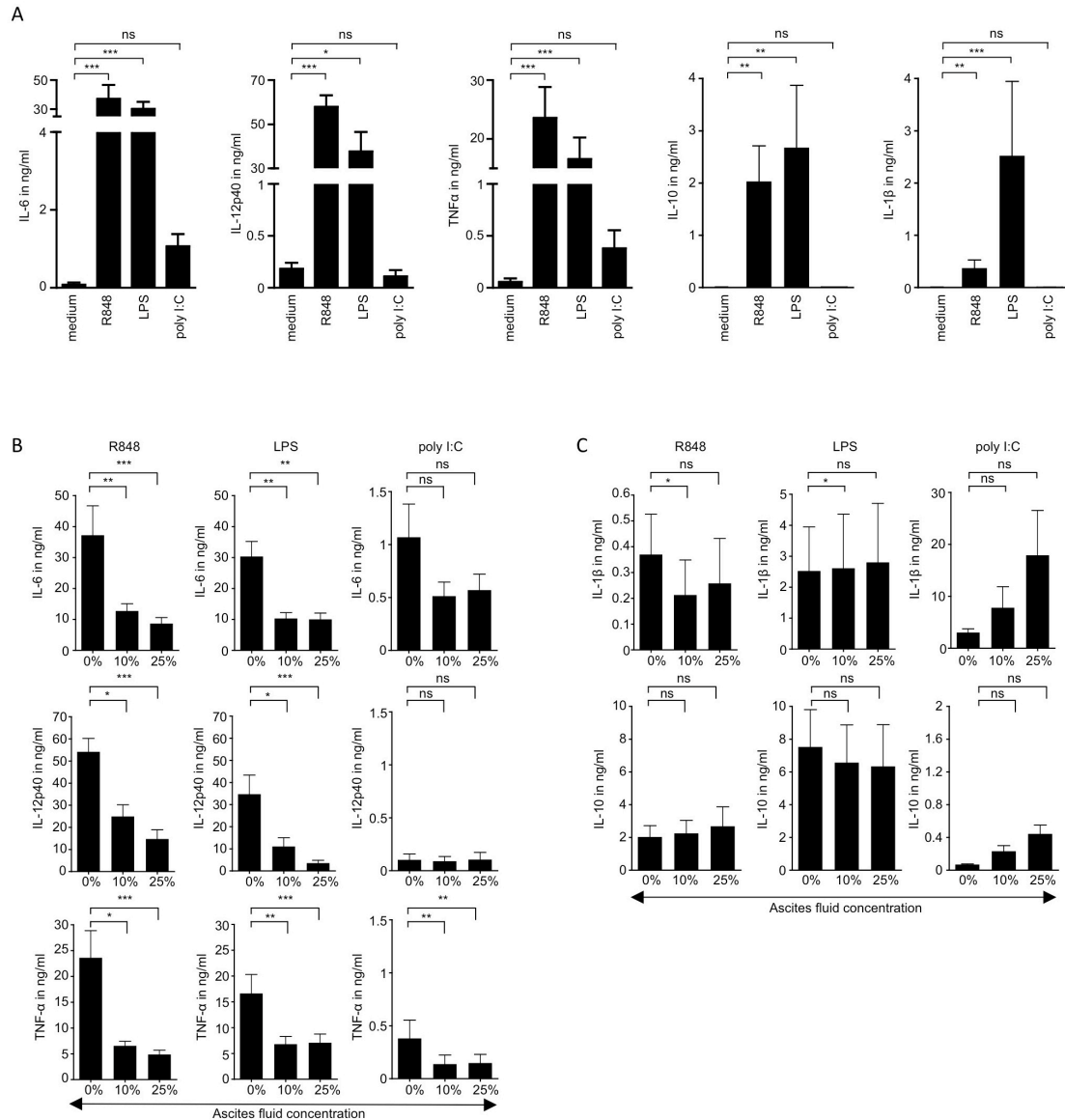


Figure 3.6 Ovarian carcinoma associated ascites partially suppresses TLR-mediated production of IL-6, IL-12 and TNF α .

(A) Monocyte-derived DC were stimulated overnight with 3 μ g/ml R848, 1 μ g/ml LPS or 100 μ g/ml polyI:C and (B-C) ascites from patients with ovarian carcinoma was either absent (0%) or added to cultures at concentrations of 10% or 25%. IL-6, TNF α , IL-1 β and IL-10 levels were measured in cell culture supernatants by bead ELISA (Flow Cytomix), IL-12p40 was measured by sandwich ELISA. For IL-6, IL-12p40 and TNF α : n=12 (DC from six different healthy volunteers were cultured with ascites from 5 different ovarian carcinoma patients. Altogether, 12 independent experiments were performed. Cells from three volunteers were cultured with only one ascites sample each, while cells from the remaining three donors were cultured with two, three or four different ascites samples, respectively). For IL-1 β and IL-10: n=9 (DC from six different healthy volunteers cultured with ascites from 5 different ovarian carcinoma patients. Altogether, 9 independent experiments were performed. Cells from four volunteers were cultured with only one ascites sample each, while cells from the remaining two donors were cultured with two or three different ascites samples, respectively). One-way ANOVA (Friedman test with Dunn post test): * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant.

TLR-mediated activation further induced the production of IL-1 β . The effect of ascites on its levels, however, varied considerably between donors, and no consistent pattern of suppression or enhanced induction in presence of ascites became evident in our study (Figure 3.6 C). We further examined levels of the immunosuppressive cytokine IL-10, since we hypothesized that in opposing fashion to the pro-inflammatory cytokines IL-6, IL-12 and TNF α , IL-10 production by DC may be enhanced by ascites to promote an immunoregulatory environment. However, similarly to IL-1 β , IL-10 levels remained unaffected by ascites (Figure 3.6 C).

3.5 Peritoneal fluid from patients with benign ovarian tumours partially suppresses TLR-mediated up-regulation of CD86 and induction of IL-6 and IL-12 but not TNF α

Our observations of a significant influence of ovarian carcinoma associated ascites on the TLR-mediated activation of monocyte-derived DC as outlined on the previous pages were intriguing and thought-provoking. However, early on in our study, we recognized the need for suitable control material to use in parallel to ascites from ovarian carcinoma patients in our experiments. More specifically, we were interested in obtaining samples of peritoneal fluid associated with non-malignant conditions in order to recognize particular characteristics of the malignant ovarian carcinoma environment. Benign ovarian tumours constitute a heterogeneous group of conditions such as fibromas, fibroadenomas, benign ovarian cysts and others. Although the formation of ascites is typically not associated with these conditions, some patients do present with a small amount of peritoneal fluid. Such samples were collected by our clinical collaborators during surgery for the removal of the patients' benign tumours of the ovary, and we were able to use such benign peritoneal fluid in our activation assays. The samples were processed identically to the malignant ascites, removing cellular material by centrifugation followed by sterile filtration before use in cell cultures.

We conducted experiments identical in their setup to those with malignant ascites, activating monocyte-derived DC from healthy donors with R848, LPS or poly I:C in absence or presence of 10% or 25% benign peritoneal fluid. Similarly to the effects seen with malignant ascites, we observed a highly significant impairment of CD86 up-regulation in presence of 25% benign peritoneal fluid when cells were activated with either of the studied TLR agonists (Figure 3.7 A). Upon activation with LPS or poly I:C, even the lower concentration of 10% peritoneal fluid in culture resulted in significantly decreased expression levels of CD86. We further observed impaired

production of the cytokines IL-6 and IL-12p40 in presence of 10% or 25% benign peritoneal fluid upon stimulation with R848 or LPS. In comparison, as shown previously (Figure 3.6 A), activation of monocyte-derived DC with poly I:C failed to induce IL-12p40 and led to considerably lower levels of IL-6 than R848 and LPS. We detected no suppression of IL-6 upon TLR3 activation in presence of benign peritoneal fluid (Figure 3.7 A).

A further parameter measured as read-out from this activation assay was TLR-mediated TNF α induction. Strikingly, we saw no suppression of this cytokine by benign peritoneal fluid after R848 or LPS activation whatsoever (Figure 3.7 B). This marked an important difference between benign peritoneal fluid and ascites from ovarian carcinoma, which, as shown above, impaired TNF α induction significantly (see above, Figure 3.6 B). Interestingly, when activated with poly I:C, 25% of benign peritoneal fluid showed statistically significant impairment of TNF α production. However, as explained previously, given the overall low levels of cytokine induced, the importance of this finding may be limited.

Taken together, the collective data from *in vitro* assays using R848, LPS and poly I:C for activation of monocyte-derived DC in absence versus presence of either ovarian carcinoma associated ascites or benign peritoneal fluid highlighted immunosuppressive properties of both the malignant and benign sample groups. An important difference that our data demonstrated was the suppression of the pro-inflammatory cytokine TNF α , which was exclusively seen in presence of malignant ascites while benign peritoneal fluid left TNF- α levels unaffected. These findings supported the notion that the environment of malignant ovarian tumours can be distinguished from that of benign conditions, and therefore may affect immunological processes in a different way.

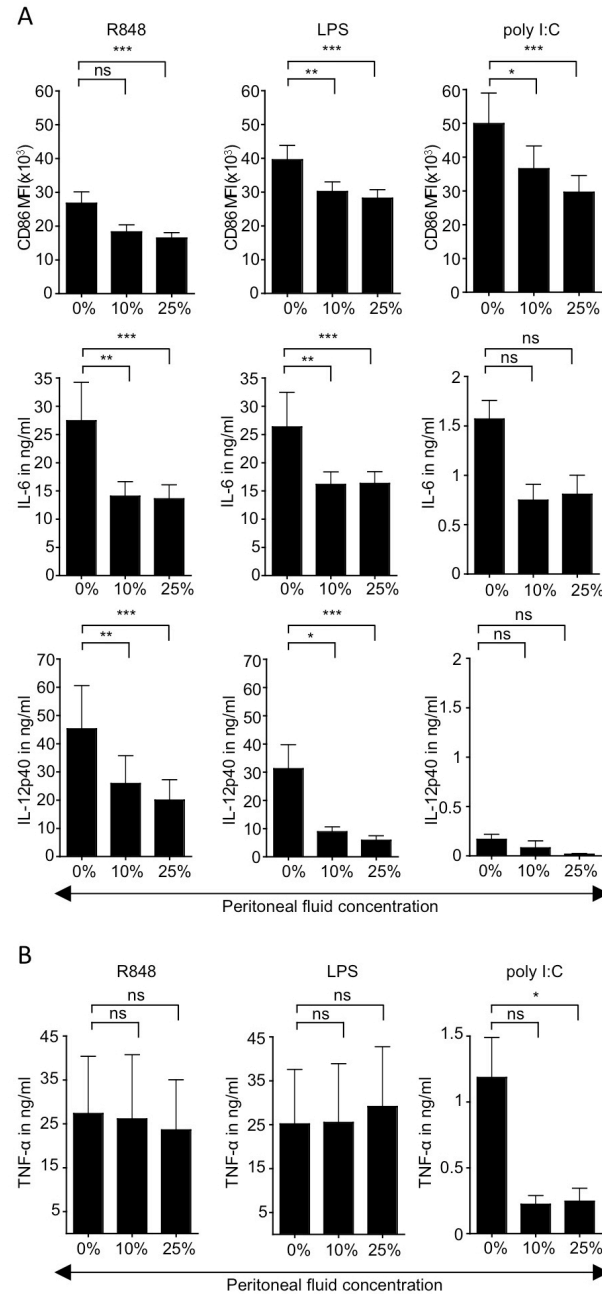


Figure 3.7 Peritoneal fluid associated with benign ovarian tumours partially suppresses TLR-mediated up-regulation of CD86 and production of IL-6 and IL-12 but not TNF α .

Monocyte-derived DC were stimulated overnight with 3 μ g/ml R848, 1 μ g/ml LPS, 100 μ g/ml polyI:C in the presence of 0%, 10% or 25% of peritoneal fluid from patients with benign ovarian tumours. **(A)** CD86 levels were assessed by flow cytometry and the cytokines IL-6, IL-12p40 and **(B)** TNF α were measured in cell culture supernatants by bead ELISA (Flow Cytomix; IL-6 and TNF α) or sandwich ELISA (IL-12p40). n=12 (DC from 8 different healthy volunteers were cultured with peritoneal fluid from 6 different patients. Altogether, 12 independent experiments were performed. Cells from four volunteers were cultured with only one peritoneal fluid sample each, while cells from the remaining four donors were cultured with two different peritoneal fluid samples); One-way ANOVA (Friedman test with Dunn post test): * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant.

Discussion

TLR mediated activation of DC subsets constitutes an important component of many experimental concepts explored in cancer immunotherapy. Notably, vaccine designs aiming to activate APC in situ within the tumour environment by targeting specific DC subsets are a promising, much studied strategy. In such settings, DC receive stimuli promoting their activation delivered by the vaccine during concomitant exposure to the tumour environment which often comprises an array of immunosuppressive factors. Understanding how DC integrate these conflicting influences is an important step towards improved protocols for in situ DC activation in future.

In this project, we explored the influence of ascites from patients suffering from advanced stage ovarian carcinoma on DC activation via TLR agonists. For reasons outlined in the introduction to this chapter, we have first chosen to use *in vitro* generated monocyte-derived DC to address our scientific questions.

When sterile filtered, cell-free ascites was added to DC cultures, we observed a significant impairment of TLR-mediated up-regulation of the co-stimulatory molecule CD86 and production of the cytokines IL-6, IL-12p40 and TNF α . Our results showed high reproducibility when monocyte-derived DC from different healthy donors were cultured with ascites from the same ovarian carcinoma patient, and equally when DC from one healthy donor were either treated with different ascites samples or, in several different experiments, repeatedly treated with the same fluid.

Interestingly, not all markers of activation were affected by the presence of ovarian carcinoma ascites, since the TLR agonist-induced increase in the expression levels of the surface markers HLA-DR and CD40 remained unchanged. Similarly, the induction of the pro-inflammatory cytokine IL-1 β and the immunosuppressive protein IL-10 were not altered by ascites. This shows that ovarian carcinoma ascites exerts its

suppressive influence on TLR-mediated DC activation with a certain specificity, interfering with distinctive pathways and leaving other unaffected.

The surface marker CD86, also referred to as B7-2, is a member of the B7 family comprising several other co-stimulatory and co-inhibitory molecules expressed by APC, most notably CD80 (B7-1) and PD-L1 (B7-H1) and PD-L2 (B7-DC), respectively (Greenwald et al., 2005). During antigen presentation, peptides are displayed on MHC molecules on the surface of APC and are sensed by TCR on T lymphocytes. An accessory signal between the protein CD28 on the surface of T cells and CD80 and/or CD86 expressed on APC is necessary for the successful priming and activation of the interacting T cell, leading to its expansion, cytokine secretion and execution of effector functions. Lack of such co-stimulation results in T cell tolerance or anergy (Reis e Sousa, 2006).

However, in order to prevent uncontrolled and excessive lymphoproliferation, engagement of co-inhibitory molecules is crucial after initial activation. Interestingly, the same molecules on APC, CD80 and CD86, can convey such abrogating signals by interacting with the co-inhibitory cytotoxic T lymphocyte antigen-4 (CTLA-4) on T cells. The nature of the signal, either co-stimulatory or co-inhibitory, is therefore dependent on both molecules participating in the interaction, and receptor promiscuity is a common trait of many B7 family members. For example, the co-inhibitory molecules PD-L1 and PD-L2 exert their functions by interaction with the receptor PD-1, but PD-L1 has recently also been shown to bind to CD80 (Greenwald et al., 2005; Keir et al., 2008).

The relative expression levels of co-stimulatory and co-inhibitory molecules on APC can influence the outcome of T cell priming, skewing it towards activation or anergy. The importance of this balance in the orchestration of an efficient immune response against tumours is evident from the promising results achieved with the blockade of the co-inhibitory checkpoints by monoclonal antibodies. Clinical trials using CTLA-4 blocking antibody Ipilimumab and PD-1 blocking antibodies Lambrolizumab and

Nivolumab in the treatment of advanced melanoma are currently underway in phase 1 (Hamid et al., 2013; Wolchok et al., 2013).

Ovarian carcinoma tumour cells reportedly express the co-inhibitory molecule PD-L1 and interact with tumour infiltrating lymphocytes (TIL), impairing their cytotoxic activity (Hamanishi et al., 2007). This mechanism has also been implicated to promote peritoneal dissemination of tumour cells and thereby accelerate disease progression (Abiko et al., 2013).

Macrophages expressing co-inhibitory molecules of the B7 family have been identified in the ovarian carcinoma environment and have been shown to suppress the initiation of effector T cell responses (Kryczek et al., 2006). A monocyte population with T cell inhibitory properties has equally been identified in ovarian carcinoma ascites, lacking cell surface expression of the co-stimulatory proteins CD80 and CD86 (Loercher et al., 1999). Surprisingly, although the physiological relevance of this is unclear, a subpopulation of TIL isolated from ovarian carcinoma ascites have been reported to express CD80 and CD86 (Melichar et al., 2000). The relevance of the expression of these molecules by T cells remains poorly understood, but some evidence from the field of transplantation suggests that through interaction with T reg, T cells expressing CD80 and CD86 downregulate alloresponses and prevent graft-versus-host-disease (GVHD) (Greenwald et al., 2005). The mechanism behind this is thought to be the reception of inhibitory signals via CD80 and CD86 from CTLA-4 expressed on T reg (Paust et al., 2004).

These reports collectively point towards presence of certain factors within the ovarian carcinoma environment that interfere with the pathways regulating the expression patterns of B7 family members in several leukocyte populations. Our findings of significantly impaired up-regulation of CD86 on monocyte-derived DC upon TLR stimulation in presence of ovarian carcinoma ascites support this notion. In a limited number of experiments, we also investigated expression levels of the co-inhibitory molecule PD-L1 on DC in absence or presence of ascites upon TLR stimulation, but

observed no consistent changes in expression levels of this molecule induced by ascites. However, it is important to note that in order to provide cells with favourable *in vitro* growth conditions, we only investigated the influence of up to 25% ascites in culture on DC activation. It is possible that this dilution of certain factors within ascites led to a reduction of their final cell culture concentration below a threshold necessary to exert influence on the levels of PD-L1.

Additionally, in our study we only addressed the influence of soluble factors within ascites on DC activation. Mechanisms that influence surface expression of B7 family members may require contact between cellular components of the tumour environment. One such process has been identified in a recent study by Qureshi et al., where the authors elegantly demonstrated that CD80 and CD86 are removed from the surface of APC and internalized by T cells via trans-endocytosis upon interaction with CTLA-4 (Qureshi et al., 2011). Similar mechanisms requiring direct cell-cell contact could be effecting other surface markers, but these questions were not addressed in our study.

The above mentioned limitations regarding exclusive use of cell-free ascites and its restricted concentration in culture could also contribute to the reasons why levels of CD40 and HLA-DR remained unaffected in our experiments. However, we did occasionally notice changes in expression levels of these two markers in presence versus absence of ascites, but these were not consistent between experiments. It is feasible that this is due to inter-individual differences between monocyte-derived DC donors, ascites samples and/or their respective combinations, but in order to recognize patterns, dissect the reasons and understand the implications thereof, much bigger experimental cohorts than available for this study would be required.

Apart from surface marker expression, we also monitored the influence of ascites on cytokine induction by monocyte-derived DC. We detected a significantly impaired production of IL-6, IL-12p40 and TNF α in presence of ascites from ovarian carcinoma

patients. The network of cytokines within the tumour environment and its impact on disease progression and prognosis has been subject to extensive study in the field of cancer immunology. Chronic inflammation is understood to be linked to carcinogenesis, and particularly in ovarian carcinoma, the cytokines IL-6 and TNF α are implicated as major drivers in this regard (Szlosarek et al., 2006; Kulbe et al., 2007; Maccio and Madeddu, 2012). However, assessing the environment of ovarian carcinoma under steady-state conditions was not the aim of our study, but rather the question whether upon TLR activation, we can achieve potent DC activation leading to a production of these pro-inflammatory cytokines. Despite their documented role in advancing disease as drivers of chronic inflammation, production of pro-inflammatory cytokines is an important component in the initiation of a potent anti-tumour response. In order to lead to differentiation of T cells into effector cells, antigen presentation and co-stimulation as described above necessitate an accompanying third signal which is conveyed by cytokines. The cytokines are produced by the activated APC and act on the engaged T cell, contributing heavily to the polarization of the resulting T cell response (Diebold, 2008). Direct activation via PRR is thought to be necessary for cytokine production (signal 3) by APC, thereby rendering the APC fully activated and capable of conveying all three signals necessary for T cell priming and differentiation (Edwards et al., 2002; Sporri and Reis e Sousa, 2005). Up-regulation of co-stimulatory molecules on APC (signal 2) does most likely not require direct PRR stimulation and can be induced by indirect activation through inflammatory mediators produced by surrounding cells (Sporri and Reis e Sousa, 2003; Joffre et al., 2009).

The cytokine IL-12 induces differentiation of naïve T cells into T_H1 cells, which are potent producers of IL-2, IFN γ , TNF α , lymphotoxin (LT), IL-3 and GM-CSF. While IL-2 stimulates lymphocyte and NK cell proliferation, IFN γ , TNF α and LT activate NK cells and macrophages, promoting their cytotoxic and phagocytic as well as antigen presenting activity, respectively. The factors IL-3 and GM-CSF act as growth factors in myelopoiesis, encouraging the production of granulocytes, macrophages and

DC in the bone marrow. Overall, a dominating T_H1 response primarily promotes cell-mediated immunity as opposed to humoral immune responses, and is therefore desirable in the context of tumour immunotherapy (Murphy et al., 2008). A suppression of IL-12 production by DC as observed in our study could reduce T_H1 induction, with unfavourable consequences for the polarization of the resulting adaptive immune response. It is important to note, however, that for T cell skewing away from T_H1 to take effect, the reduction of IL-12 may have to be accompanied by concomitant presence of cytokines and/or co-stimulatory molecules inducing other types of T_H cell responses.

A further factor that was potentially induced by TLR activation of monocyte-derived DC but strongly suppressed in presence of ascites in our experiments was IL-6. This cytokine has equally been described to influence the direction of the induced T cell response following antigen presentation, in particular effecting the balance between promotion of T regs and T_H17 cells (Manel et al., 2008; Volpe et al., 2008; Kimura and Kishimoto, 2010). The balance of T reg versus T_H17 responses bears prognostic value in ovarian carcinoma, with high T reg numbers in the tumour environment being associated with poor clinical outcomes (Curiel et al., 2004; Sato et al., 2005) and T_H17 being linked to prolonged survival and therefore considered favourable (Kryczek et al., 2009). In contrast, in the context of autoimmune diseases such as rheumatoid arthritis (RA), the opposite ratio is desirable, with T regs having a protective role and T_H17 driving pathological inflammation. IL-6 blockade has yielded positive results in experimental treatment of RA patients, increasing their T reg numbers in peripheral blood and thereby skewing the ratio of T reg/T_H17 cells towards a more regulatory T cell response (Pesce et al., 2013). Our results in this study show that TLR-induced IL-6 production by DC is significantly impaired in presence of ovarian carcinoma ascites. It is difficult to extrapolate these observations to the effects they may have on the T cell response, because the latter is orchestrated by many additional factors. However, providing the presence of other soluble factors such as TGF β or IL-10, one

may hypothesize that reduced IL-6 levels could contribute towards an increased T_{reg}/T_H17 ratio, which may be of disadvantage for immunotherapeutic concepts in ovarian carcinoma. A further cytokine that promotes T_H17 induction is IL-1 β (Sutton et al., 2006), hence we investigated the influence of the ovarian carcinoma environment on IL-1 β production by DC but did not see consistent alteration of this cytokine in presence of malignant ascites.

TNF α is a cytokine first discovered for its ability to induce necrosis of tumour tissue (Carswell et al., 1975) and it has since been shown to have multiple functions in the immune system, acting as a pyrogen, vasodilator and initiator of the acute phase response during infection. TNF α induces the production of an array of pro-inflammatory cytokines such as IL-6 and IL-1 β and in systemic infections, circulating TNF α has been identified as a pivotal factor inducing cachexia by promoting lipolysis and interfering with other metabolic processes (Beutler et al., 1985). In acute sepsis, effects of high TNF α levels can be detrimental, leading to complications such as cytokine storm, hypotensive crisis through systemic vasodilatation, coagulopathy and ultimately multiple organ failure and death. The importance of TNF α as a causative factor for these symptoms has been implicated by animal models where partial protection of otherwise fatal infections was achieved by preventive administration of TNF α blocking agents (Lorente and Marshall, 2005). However, the benefits of TNF α blockade in sepsis have not been consistent throughout pre-clinical studies, and seem to depend on multiple factors including type of pathogen and host species used in the study (Lorente and Marshall, 2005). Equally, the translation of TNF α blockade into clinical use in sepsis in humans has only shown limited success, but clinical trials using TNF α blockers for treatment of this condition are nevertheless ongoing, with the aim of gaining a better understanding of the complexity of the clinical inflammatory response (Qiu et al., 2011).

In contrast, the use of TNF α inhibitors such as etanercept or infliximab has gained tremendous success in the therapy of RA (Upchurch and Kay, 2012). In the context of

the localized synovial inflammation present in RA, TNF α promotes recruitment of monocytes, neutrophils and lymphocytes to these sites by inducing vasodilatation and expression of adhesion molecules on endothelial cells of the local vasculature. It also induces the production of pro-inflammatory cytokines, sustaining an inflammatory environment and thereby fostering disease progression. RA patients who benefit from anti-TNF α therapy are subjected to continuous use of these biologicals over many years, and side effects of these therapeutic regimes have highlighted the importance of TNF α in the control of latent infections such as tuberculosis or hepatitis, with documentedly increased risk of exacerbation of these diseases during anti-TNF α therapy (Rubbert-Roth, 2012). Long-term use of TNF α inhibitors is being debated as a potential risk factor for the development of malignancies and remains subject to controversy. Thus far, this notion is not conclusively supported by the vast majority of clinical data available (Rosenblum and Amital, 2011; Park and Ranganathan, 2012), possibly with the exception of non-melanoma skin cancer (Le Blay et al., 2012).

TNF α has been implicated to be a central regulator of the pro-inflammatory milieu in ovarian carcinoma, sustaining a cytokine and chemokine network including IL-6, VEGF α , CCL2 and CXCL12 and thereby promoting pelvic inflammation and tumour dissemination and progression (Kulbe et al., 2007). It has been shown that malignant ovarian epithelium produces higher levels of TNF α as compared to normal tissue, and ovarian carcinoma cells express the TNF α receptor TNFR1 (Szlosarek et al., 2006). Within the tumour environment, stromal cells as well as various leukocyte populations such as macrophages and DC are a further important source of TNF α .

In this study, we have observed substantial production of TNF α by monocyte-derived DC upon stimulation with TLR agonists, and the levels of this cytokine were significantly reduced when activation took place in presence of ovarian carcinoma associated ascites. In the context of our study, these data once again show immunosuppressive properties of the ovarian carcinoma tumour environment, because in the context of an acute immune response, TNF α is required for maturation of DC

and their capacity to migrate to lymphnodes where priming of naïve T cells takes place (Banchereau and Steinman, 1998; Stoitzner et al., 1999). Hence, TNF α is indirectly crucial for the initiation of anti-tumour responses by DC (Miwa et al., 2012).

Interestingly, when comparing ascites from ovarian carcinoma patients to peritoneal fluid associated with benign ovarian tumours with regards to their respective effects on TLR-mediated activation of monocyte-derived DC, we observed a striking difference in TNF α suppression. Benign peritoneal fluid left TNF α production induced by TLR stimuli unaffected, while malignant ascites robustly suppressed the production of this cytokine. Other monitored markers of activation, namely IL-6, IL-12p40 and the surface marker CD86 were equally suppressed in presence of both the benign and malignant fluids. Hence, while this points towards similarities in composition of the soluble factors within the environment of benign and malignant ovarian tumours, important differences remain and should be explored in more depth, as they may lead to a better understanding of particularities of malignant milieus that distinguish them from benign conditions.

With specific regard to our study, the pathways leading to TNF α production upon TLR stimulation in monocyte-derived DC were differentially influenced by soluble factors present within the environment of malignant versus benign ovarian tumours. As outlined above, TNF α promotes the recruitment of leukocyte subsets to the tumour environment, is important for DC maturation and migration and through these and other functions contributes to the creation of a pro-inflammatory environment. A suppressed induction of TNF α upon TLR activation may therefore hinder efficient initiation of immune responses and may represent an important obstacle in experimental concepts relying on in situ DC activation in ovarian carcinoma.

In order to gain a better understanding of the mechanisms underlying the immunosuppressive properties of the ovarian carcinoma environment, an investigation of the constitution of ascites represented the next step in our study. We further explored the contribution of individual ascites components to the impairment of TLR-

mediated monocyte-derived DC activation, and our respective findings are outlined in detail in the following chapters.

Chapter 4

Investigating components of ovarian carcinoma associated ascites that impair TLR-mediated monocyte-derived DC activation

Introduction

Regardless of the underlying pathophysiological causes leading to formation of ascites, this material harbours a large array of proteins, many of which have numerous functions in the human immune system. As several studies have revealed, ovarian carcinoma fosters an immunosuppressive tumour environment comprising many cytokines and chemokines with known immunosuppressive properties. These factors are produced by tumour cells themselves and/or leukocytes infiltrating the tumour, can be found in the immediate tissue surroundings and are consequently also present in ascites from patients suffering from ovarian carcinoma (Yigit et al., 2010; Wertel et al., 2011; Yigit et al., 2011a; Yigit et al., 2011b). A review of available studies characterizing the ovarian carcinoma tumour environment revealed considerable differences between absolute levels of different proteins as well as large variations between ascites samples from different individuals. This is only natural, given the dynamic nature of tumour tissue, frequent migration of leukocyte populations and the innumerable plethora of other factors that collectively determine the precise composition of the tumour environment at any given timepoint. The consistency and reproducibility of the suppressive effects of malignant ascites on TLR-mediated activation of DC as observed in our study and presented in the previous chapter was therefore all the more remarkable. The findings indicated that despite their inevitable differences, some similarities between the individual ascites samples used in our

experiments do exist. Exploring these similarities further could lead to the discovery of particular factors that act as obstacles hindering TLR-mediated activation of DC. Once the mechanisms underlying the observed suppression are understood, they could be tackled and potentially overcome.

A simple and efficient way to explore the role of a protein in any given scenario is its selective blockade or neutralization. Historically, immunoglobulins were discovered as substances in human serum that had the ability to neutralize bacteria and other ‘toxins’. They were therefore first referred to as anti-toxins before Paul Ehrlich proposed the term antibody which was deemed more accurate and appropriate with the finding that ligands of immunoglobulins are not strictly toxins (Lindenmann, 1984). Koehler and Milstein reported in 1975 that a virtually endless supply of monoclonal antibodies of any desired specificity could be produced with the help of hybridoma cell lines, which was a landmark in scientific research (Kohler and Milstein, 1975). Nowadays, generation of antibodies against any defined antigen is possible, and countless scientific applications rely on the use of immunoglobulins. They can be designed to fulfill an array of functions, among which is the selective neutralization of their particular ligands. Neutralization typically leads to the abrogation of the biological activity of the specific ligand, and this approach lends itself to studies exploring the biological function of particular agents.

Due to the convincing reproducibility of our experiments where ascites from different ovarian carcinoma patients consistently suppressed TLR-mediated DC activation, we hypothesized that a common component of all ascites samples predominantly induced the observed suppressive effect. An array of carefully selected antibodies specifically neutralizing particular proteins in our cell cultures was therefore an ideal tool for our study, allowing us to dissect the contribution of individual factors in ascites to the suppression of TLR-mediated DC activation. This chapter describes our attempts to tackle these exciting and highly relevant scientific questions and reveals the insights

we have gained into the composition and attributes of the ovarian carcinoma ascites environment.

4.1 Composition of ovarian carcinoma associated ascites

In order to identify the factors contributing to the observed suppressive effects of ascites from ovarian carcinoma patients on TLR-mediated DC activation, it was important to first elucidate the composition of the malignant ascites. Previous reports have analyzed protein levels in ascites samples from ovarian carcinoma patients (Giuntoli et al., 2009b; Yigit et al., 2011b) and have found an array of pro-inflammatory and immunosuppressive cytokines and chemokines present at varying levels. To understand the composition of the material used in this study, we analyzed all malignant ascites samples for levels of the immunosuppressive proteins IL-10, leukemia inhibitory factor (LIF), arginase-1 (Arg-1) and prostaglandin E₂ (PGE₂). We further determined the concentrations of the angiogenesis-promoting and immunosuppressive factor VEGF α , chemokine ligand 18 (CCL18), and the pro-inflammatory cytokines IL-6, TNF α and IFN γ . All measured proteins were present in detectable levels in at least one ascites sample (Figure 4.1), with the exception of soluble CTLA-4 (sCTLA-4) which could not be detected in any of our samples (data not shown). Most proteins measured were present in the majority of samples, although their levels varied considerably. By assigning one symbol throughout all graphs depicted in Figure 4.1 to each of the 8 ascites samples that were used throughout this study, we aim to demonstrate that each ascites sample is of a unique composition, harboring different levels of various proteins which together orchestrate an immune environment specific for the tumour environment of each individual patient.

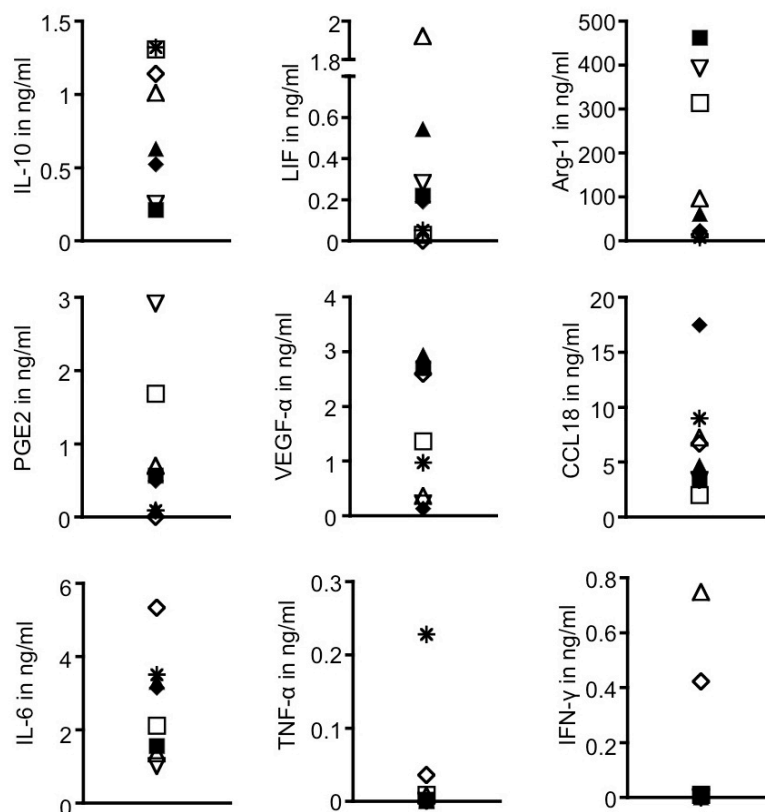


Figure 4.1 Levels of proteins in ovarian carcinoma associated ascites vary considerably between individuals.

Protein levels in ascites samples collected from ovarian carcinoma patients were measured by sandwich ELISA. Eight malignant ascites samples used throughout the study are shown. Each symbol represents one patient sample throughout the graphs. $n = 8$.

Levels of IL-10, LIF, VEGF- α , CCL18, IL-6, TNF α and IFN- γ were measured by Hayley Evans or Ann Jagger (research group of Dr. Leonie Taams, Centre for Molecular & Cellular Biology of Inflammation, King's College London).

4.2 Correlation between protein levels in ascites and suppression of TLR-mediated monocyte-derived DC activation

Our experimental results thus far have shown suppression of a number of markers of TLR-mediated monocyte-derived DC activation. Although the suppression was consistent across ascites samples from different patients, we noticed differences in the intensity of suppression of our read-out markers CD86 and cytokines IL-6, IL-12p40 and TNF α . We were interested to explore whether this was due to experimental variation or whether the composition and levels of individual factors detected in our ascites samples may have influenced the suppressive activity of each particular sample. We therefore correlated levels of all measured proteins in individual ascites samples with the suppression of CD86 up-regulation on monocyte-derived DC as well as IL-6, IL-12p40 and TNF α production after R848 activation in presence of 10% ascites in cell culture as compared to R848-activated samples without ascites. The formula used to calculate the suppression was as follows:

$$\frac{(\text{MOA}_{[\text{R848} + \text{no ascites}]} - \text{MOA}_{[\text{R848} + 10\% \text{ ascites}]})}{\text{MOA}_{[\text{R848} + \text{no ascites}]}} \times 100 = \text{suppression in per cent (\%)}$$

MOA = marker of activation (CD86 MFI values or IL-6, IL-12p40 or TNF α levels)

When suppression of CD86 up-regulation by individual ascites samples was paired with levels of the cytokine IL-10 in the respective samples, we observed a strong positive correlation, i.e. the more IL-10 present in an ascites sample, the stronger was the suppression of surface marker CD86 induced by this particular fluid (Figure 4.2). We detected the same positive correlation between IL-10 levels in ascites and suppression of IL-6 and IL-12p40. The lack of statistical significance of the correlation between IL-10 and IL-12p40 suppression was due to one outlying value, where an

ascites sample with comparably low IL-10 levels induced a suppression of IL-12p40 production over 90%. Strikingly, although TNF α production was also impaired in presence of malignant ascites (see chapter 3, Figure 3.6 B), the magnitude of suppression did not correlate with IL-10 levels in ascites (Figure 4.2, bottom right graph).

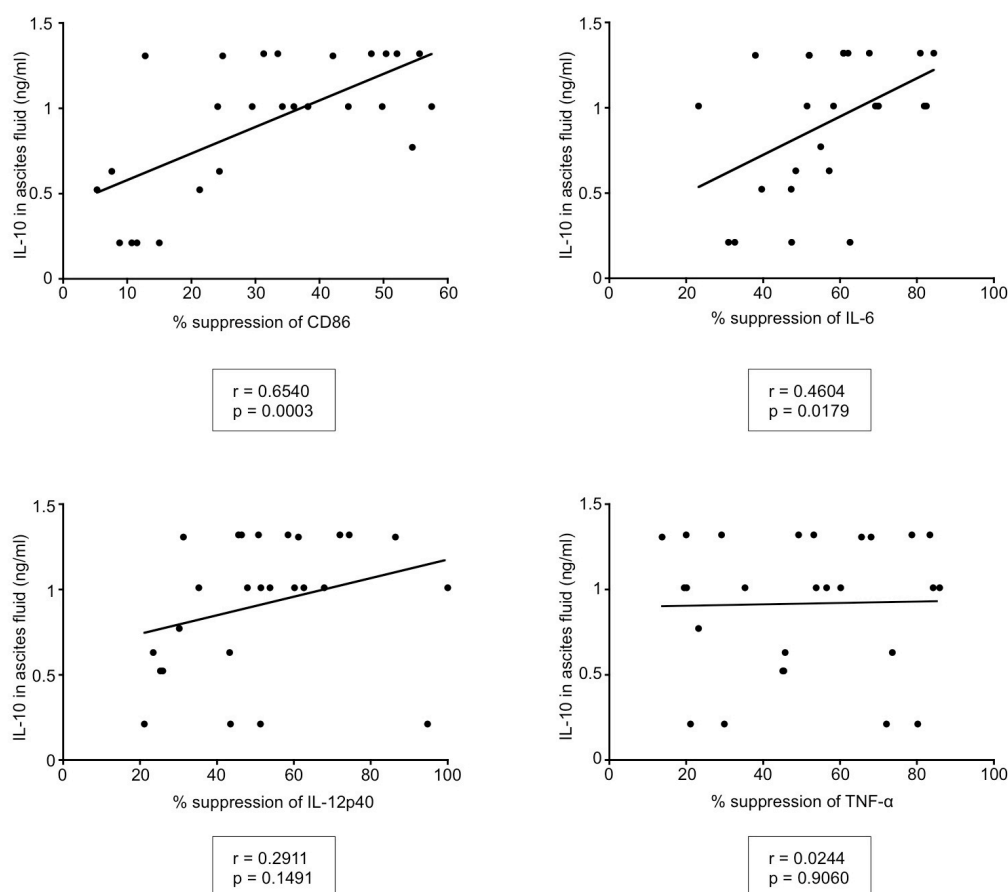


Figure 4.2 IL-10 in ovarian carcinoma associated ascites correlates positively with the suppression of CD86, IL-6 and IL-12p40, but not TNF α .

Levels of IL-10 in ascites samples are correlated to the suppression of TLR-mediated up-regulation of CD86 and production of IL-6, IL-12p40 and TNF α . Suppression is expressed in per cent reduction of surface marker or cytokine levels when 10% ascites was added to the cell culture as compared to no ascites present; $n = 26$ (DC from 9 different healthy volunteers were cultured with ascites from 8 different ovarian carcinoma patients. Altogether, 26 independent experiments were performed. Cells from four volunteers were cultured with two ascites samples each, while cells from two donors were cultured with three different ascites samples each, and the remaining three donors' cells were cultured with four different ascites samples each. The Pearson coefficient was calculated to assess statistical significance of correlations.

Levels of IL-10 in ascites samples were measured by Hayley Evans or Ann Jagger (research group of Dr. Leonie Taams, CMCBI, King's College London).

The levels of most other determined proteins found in OC-associated ascites did not correlate with the degree of its suppressive properties (Table 4.1). However, we observed statistically significant negative correlations between the levels of VEGF α and suppression of CD86 induction, as well as between Arg-1 and CD86 suppression, and Arg-1 and inhibition of IL-6 production (Figure 4.3 A-B). Although the statistical analysis suggests a positive correlation between TNF α levels in ascites and suppression of CD86, IL-6 and IL-12p40, given that only one sample contained detectable levels of TNF α (see above, Figure 4.1), this correlation is likely to be skewed and should, therefore, be disregarded (Figure 4.3 C).

	Cytokine in AF	r coefficient	p value
CD86 suppression vs.	LIF	-0.00175	0.9932
	Arg-1	-0.5513	0.0035**
	PGE ₂	0.005951	0.9770
	VEGF α	-0.5524	0.0034**
	CCL18	0.3364	0.0929
	IL-6	0.01757	0.9321
	TNF α	0.5845	0.0017**
	IFN γ	0.1554	0.4483
IL-6 suppression vs.	LIF	0.1313	0.5225
	Arg-1	-0.4527	0.0202*
	PGE ₂	-0.1950	0.3397
	VEGF α	-0.3225	0.1081
	CCL18	0.3652	0.0666
	IL-6	0.1497	0.4654
	TNF α	0.4775	0.0136*
	IFN γ	0.05195	0.8010
IL-12p40 suppression vs.	LIF	0.028	0.8920
	Arg-1	0.1047	0.6109
	PGE ₂	0.1323	0.5193
	VEGF α	-0.08265	0.6881
	CCL18	-0.0252	0.9027
	IL-6	-0.1421	0.4885
	TNF α	0.4470	0.0221*
	IFN γ	0.2443	0.2291
TNFα suppression vs.	LIF	0.08681	0.6733
	Arg-1	0.01104	0.9573
	PGE ₂	-0.07281	0.7237
	VEGF α	0.09525	0.6435
	CCL18	-0.0287	0.8893
	IL-6	0.08832	0.6679
	TNF α	0.03785	0.8544
	IFN γ	-0.01039	0.9598

Table 4.1 Correlation of cytokine levels in ovarian carcinoma associated ascites with the suppressive activity of individual ascites samples as assessed by suppression of TLR-mediated CD86 up-regulation and production of the cytokines IL-6, IL-12p40 or TNF α .

Distribution of cytokine levels in ascites samples was assessed by d'Agostino and Pearson omnibus normality test. For normally distributed cytokine levels (Arg-1, VEGF α , IL-6), the Pearson correlation coefficient was calculated. For cytokines lacking normal distribution of levels between ascites samples (LIF, PGE₂, CCL18, TNF α , IFN γ), the Spearman correlation coefficient was calculated. Pearson r or Spearman r coefficient; two-tailed; * = p<0.05 ** = p<0.01. Statistically significant correlations are shaded grey, and respective graphs are depicted below in Figure 4.3.

Levels of LIF, VEGF α , CCL18, IL-6, TNF α and IFN γ in ascites samples were measured by Hayley Evans or Ann Jagger (research group of Dr. Leonie Taams, CMCBI, King's College London).

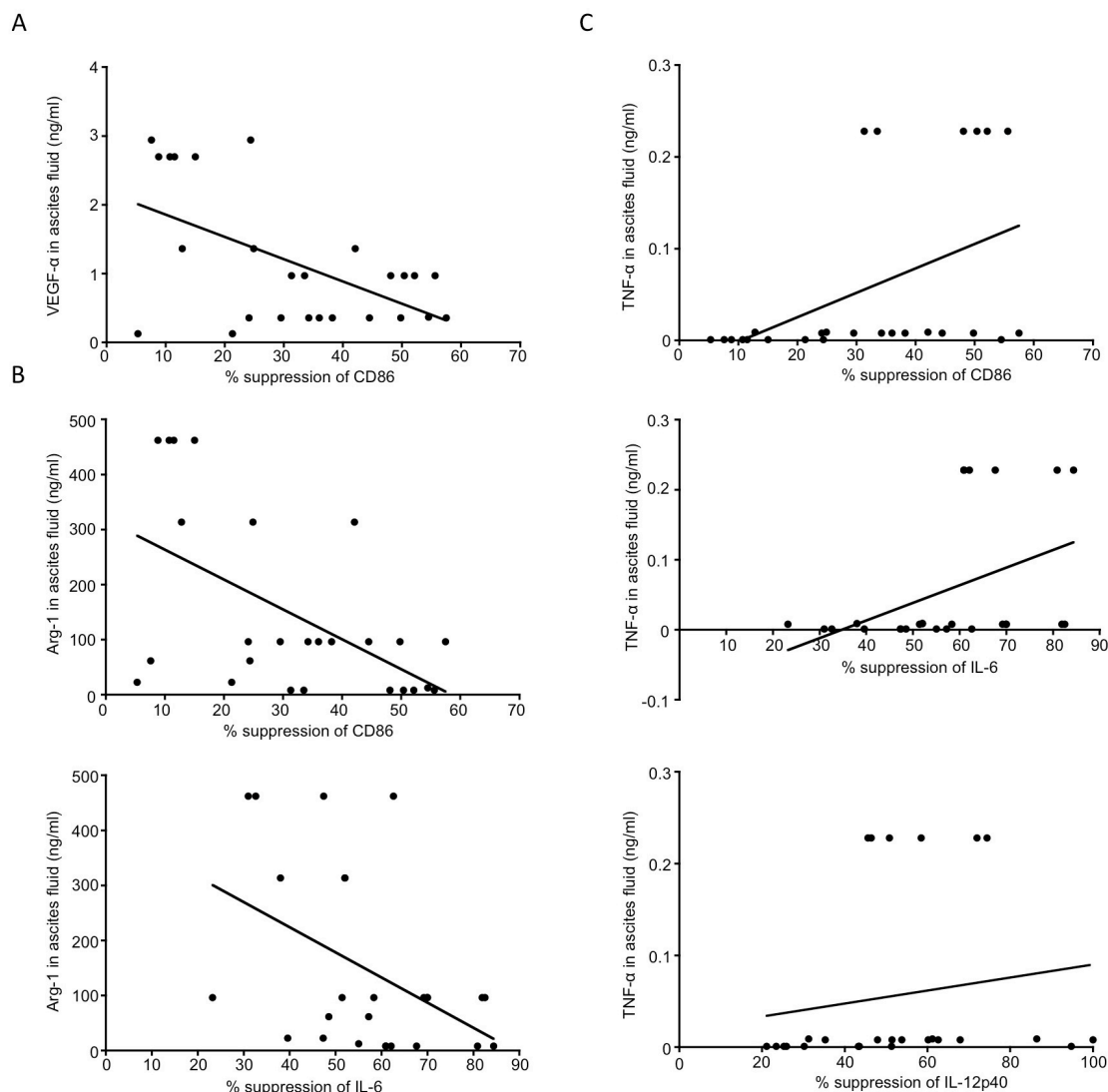


Figure 4.3 Correlation of levels of VEGF α , Arg-1 and TNF α in ovarian carcinoma associated ascites with the suppression of TLR-mediated CD86 up-regulation and/or production of the cytokines IL-6, IL-12p40.

Suppression is expressed in per cent reduction of surface marker and cytokine levels when 10% ascites was added to the cell culture as compared to absence of ascites. **(A)** Correlation between VEGF α levels and CD86 suppression: Pearson $r = -0.5524$ $p = 0.0034$ **(B)** Correlation between Arg-1 levels and CD86 suppression: Pearson $r = -0.5513$ $p = 0.0035$; correlation between Arg-1 levels and IL-6 suppression: Pearson $r = -0.4527$ $p = 0.0202$ and **(C)** Correlation between TNF α levels and CD86 suppression: Spearman $r = 0.5845$ $p = 0.0017$; correlation between TNF α levels and IL-6 suppression: Spearman $r = 0.4775$ $p = 0.0136$; correlation between TNF α levels and IL-12p40 suppression: Spearman $r = 0.4470$ $p = 0.0221$

Levels of VEGF α and TNF α in ascites samples were measured by Hayley Evans or Ann Jagger (research group of Dr. Leonie Taams, CMCBI, King's College London).

4.3 Neutralization of IL-10 in cell cultures alleviates the ascites-induced suppression of R848-mediated activation of monocyte-derived DC

The analysis of correlations between various protein levels in ascites and magnitude of suppression of monocyte-derived DC activation upon TLR8 stimulation suggested an important role for the immunosuppressive cytokine IL-10 in our experimental scenario. To explore its contribution to the suppression further, and to also examine the possible role of other proteins that have not necessarily shown a dose-dependent correlation with degree of suppression, we conducted experiments using specific neutralizing antibodies for an array of selected cytokines with known immunosuppressive properties. All cytokines that we neutralized in the following set of experiments were detected in our malignant ascites samples (see above, Figure 4.1) with the exception of TGF β which we had not measured. We nevertheless decided to include a neutralizing antibody for TGF β in our experiments, because the presence of this cytokine in ovarian carcinoma associated ascites has been reported previously (Giuntoli et al., 2009b; Yigit et al., 2011b) and it is further known to suppress DC activation (Flavell et al., 2010). In addition to IL-10 and TGF β , we neutralized the immunosuppressive cytokines LIF and VEGF α , as well as IL-6. Although IL-6 is considered a rather pro-inflammatory agent in many settings, it has been reported to exert immunosuppressive effects within ovarian carcinoma (Dijkgraaf et al., 2012) and other tumour environments by constitutive activation of signal transducer and activator of transcription 3 (STAT3) (Nguyen-Pham et al., 2012), which can lead to impairment of immune cell function, including DC activation (Yu et al., 2007).

For reasons of practicability of our assays in terms of sample and cell numbers, we chose to initially study the effects of selective cytokine neutralization on DC activation in presence of ascites using one TLR agonist only. The TLR7/8 agonist R848 showed most consistent induction of high levels of CD86 as well as IL-6, IL-12p40 and TNF α ,

and the levels of both CD86 and all three cytokines were significantly suppressed in presence of 10% ovarian carcinoma ascites (see chapter 3, Figures 3.4 and 3.6).

An identical setup to previous experiments was followed, with overnight activation of monocyte-derived DC with the TLR7/8 agonist R848 (3 $\mu\text{g/ml}$) in absence or presence of ovarian carcinoma associated ascites at a final concentration of 10% in cell cultures. In addition, neutralizing antibodies were added to selected samples, either alone or in combination of all five neutralizing antibodies together. We conducted initial titration experiments (Figure 4.4) testing two different concentrations per antibody, which were carefully chosen according to the manufacturer's data sheets while also taking into account the respective concentrations of cytokines as determined in our ascites samples (see above, Figure 4.1). In these initial experiments, we observed a partial alleviation of ascites-induced IL-6 suppression and a complete restoration of CD86 up-regulation and IL-12p40 and TNF α induction where $\alpha\text{IL-10}$ neutralizing antibody was added to cell cultures. This effect was antibody-dose dependent, with the higher concentration of 1 $\mu\text{g/ml}$ $\alpha\text{IL-10}$ antibody leading to superior levels of all cytokines. None of the other neutralizing antibodies showed any effect on DC activation levels. After these initial experiments, in order to make the subsequent assays more practicable and to also account for potential saturation of $\alpha\text{IL-10}$ antibody at the concentration of 1 $\mu\text{g/ml}$, we consistently used the concentration of 5 $\mu\text{g/ml}$ for all antibodies in subsequent experiments. Referring back to the manufacturer's recommendations, with the exception of TGF β , this concentration exceeded the required dose to ensure complete neutralization of the respective cytokines. We had not measured TGF β levels in ascites samples in our study, but the provided data sheets suggest that the use of 5 $\mu\text{g/ml}$ $\alpha\text{TGF}\beta$ neutralizing antibody ensures a neutralization of more than 90% when TGF β is present at a concentration of 0.25 ng/ml. Although absolute cytokine levels vary between samples and may even vary from study to study depending on assays used to determine TGF β concentrations, the average levels found in 35 ascites samples from advanced stage ovarian carcinoma patients in a previous

report were approximately 2.5 ng/ml (Yigit et al., 2011a). Given that we used ascites at a final concentration of 10% in cell cultures, TGF β levels could be expected to be around 0.25 ng/ml, and the use of 5 μ g/ml α TGF β neutralizing antibody should therefore largely ensure the neutralization of this protein in our assays.

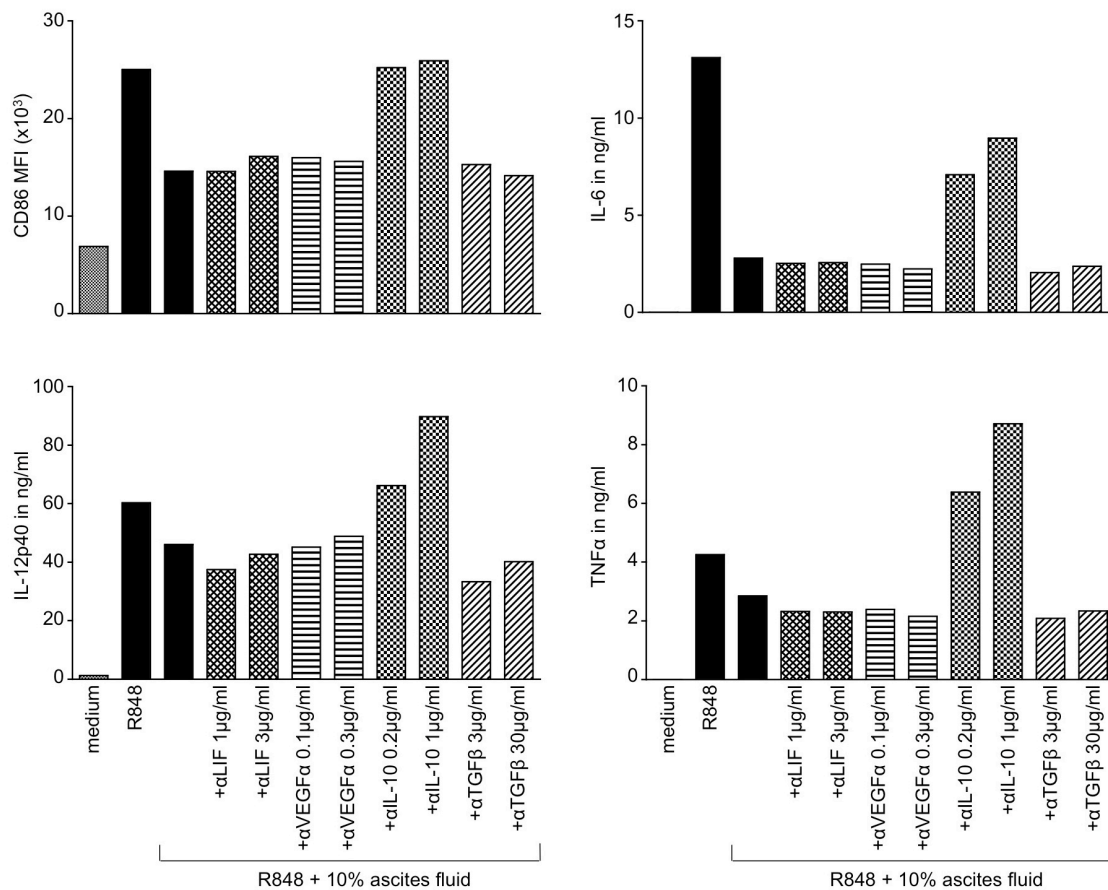


Figure 4.4 Titration of specific neutralizing antibodies in cell cultures of R848-activated monocyte-derived DC and ovarian carcinoma associated ascites.

Monocyte derived DC were cultured in the presence of 3 μ g/ml R848, 10% ascites and neutralizing antibodies at two different concentrations as indicated. CD86 expression levels were measured by flow-cytometry, and cytokine levels were measured in cell culture supernatants by Flow Cytomix analysis (IL-6 and TNF α) or sandwich ELISA (IL-12p40). This assay was performed only once as a pilot experiment in order to determine optimal antibody concentrations (n=1).

In the following set of experiments, we saw results in accordance with those obtained in the pilot experiment. Both the expression levels of co-stimulatory molecule CD86 as well as the production of the cytokines IL-6, IL-12p40 and TNF α remained unaffected when either LIF, VEGF α , TGF β or IL-6 were neutralized in cell cultures with respective antibodies (Figure 4.5). However, where IL-10 neutralizing antibody was added, monocyte-derived DC activation was restored to (IL-12p40) or even elevated beyond (CD86, IL-6 and TNF α) the levels of R848-induced activation in absence of ascites. This observed effect was robust and reproducible when using monocyte-derived DC from different donors as well as ascites samples from several ovarian carcinoma patients, and was therefore statistically highly significant. Addition of all neutralizing antibodies in combination also restored DC activation, and this effect is likely to be driven mainly by IL-10 neutralization, because the levels of activation did not differ significantly between samples where only IL-10 or all cytokines together were neutralized. IL-10 therefore emerged as a pivotal factor impeding TLR-mediated activation of monocyte-derived DC in presence of ovarian carcinoma associated ascites *in vitro*, and its neutralization alleviated the suppressive effects mediated by malignant ascites.

It is important to note that upon R848 stimulation, monocyte-derived DC produce IL-10. When adding neutralizing antibodies to cell cultures, such autocrine IL-10 is neutralized alongside ascites-derived IL-10. It is therefore not possible to exclude an effect of autocrine IL-10 on monocyte-derived DC activation under these experimental conditions. As pointed out above, where IL-10 was neutralized in cell cultures, levels of the activation markers CD86, IL-6 and TNF α were restored beyond baseline levels of cells activated with R848 alone in absence of ascites (Figure 4.5). This discrepancy pointed towards a role of autocrine IL-10 in suppression of these three markers. Interestingly, IL-12p40 levels were restored to baseline levels only, suggesting that IL-12p40 suppression is affected by ascites-derived but not autocrine IL-10.

A detailed study of the relative contributions of autocrine versus ascites-derived IL-10 to the suppression of TLR-mediated DC activation in our experiments was extensively addressed in this project, and the approaches we used to elucidate these questions are described in detail in Chapter 5.

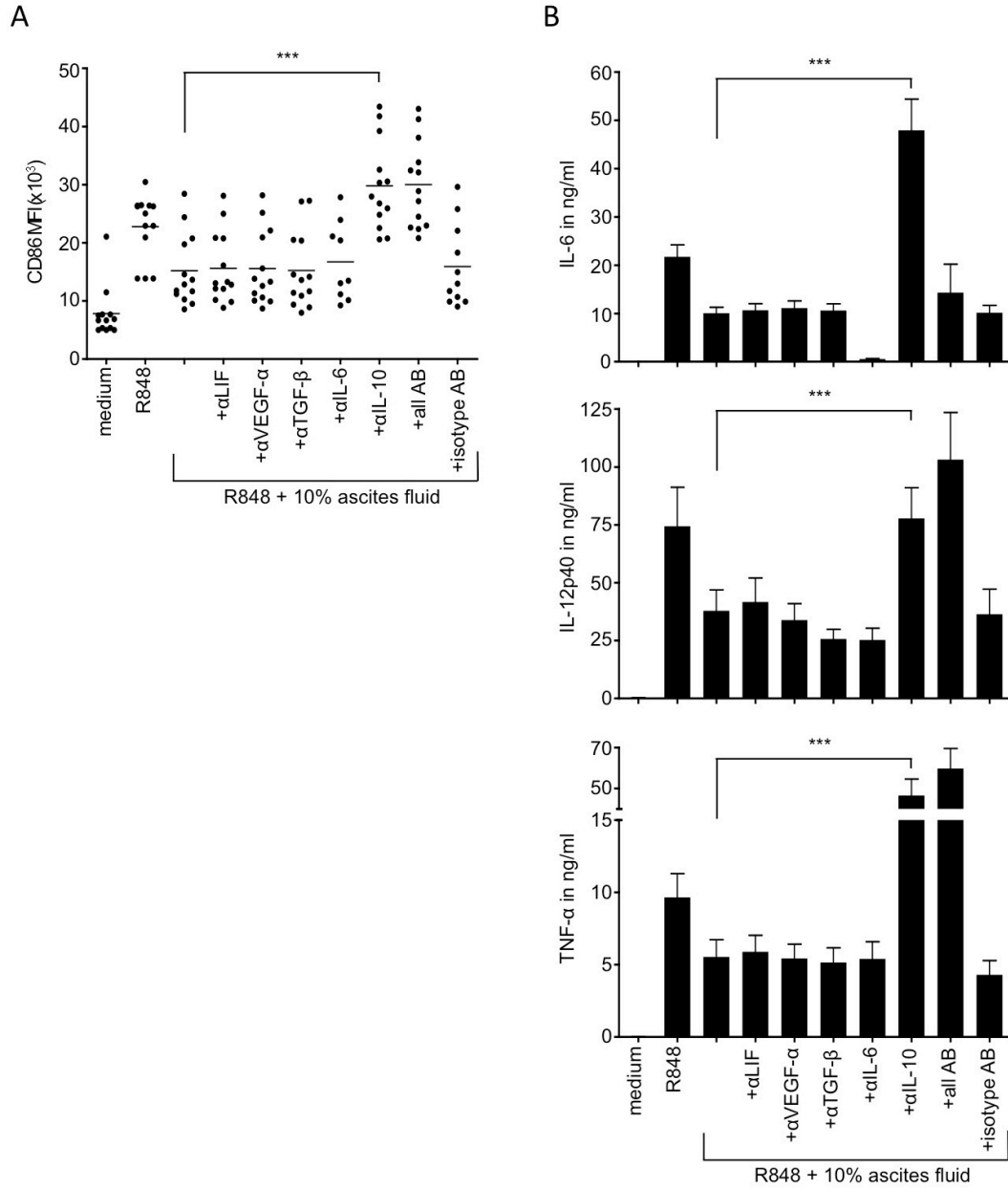


Figure 4.5 Neutralization of IL-10 in cell cultures alleviates the suppression of R848-mediated monocyte-derived DC activation.

Monocyte derived DC were cultured in the presence of 3µg/ml R848, 10% ascites and neutralizing antibodies (5µg/ml) as indicated. Neutralizing antibodies were added to cultures concomitantly with R848 and ascites, and were added alone or in combination as indicated. **(A)** CD86 expression levels were measured by flow-cytometry, and **(B)** cytokine levels were measured in cell culture supernatants by Flow Cytomix analysis (IL-6 and TNFα) or sandwich ELISA (IL-12p40). n=9-13 (DC from 8 different healthy volunteers were cultured with ascites from 7 different ovarian carcinoma patients. Altogether, 13 independent experiments were performed. Cells from three volunteers were cultured with only one ascites sample each, while cells from two donors were cultured with three, and cells from one donor were cultured with four different ascites samples, respectively. IL-6 neutralizing antibody was only used in 9 out of 13 experiments). One-way ANOVA (Friedman test with Dunn post test): *** = p<0.001. Please note that the quantification of IL-6 in samples containing IL-6 neutralizing antibody (αIL-6 and all AB) is compromised.

4.4 Neutralization of IL-10 restores LPS- but not poly I:C-mediated monocyte-derived DC activation in presence of ovarian carcinoma associated ascites

As shown above, our findings pointed towards a central role of IL-10 as an important suppressive factor impairing R848-mediated activation of monocyte-derived DC in presence of malignant ascites. We were intrigued to explore whether IL-10 was equally important in the suppression of DC activation upon stimulation with TLR4 agonist LPS and TLR3 agonist poly I:C. We therefore performed a small number of experiments using the identical panel of neutralizing antibodies as above, selectively blocking the factors LIF, VEGF α , IL-6, TGF β and IL-10 in overnight cultures of monocyte-derived DC stimulated with LPS or poly I:C. Similarly to R848, in LPS-activated cells, we observed an alleviation of suppression of CD86 up-regulation where IL-10 was neutralized, while, interestingly, cells stimulated with poly I:C showed no alteration of CD86 levels in presence of α IL-10 neutralizing antibody (Figure 4.6 A). None of the other neutralizing antibodies affected CD86 levels either in LPS- or in poly I:C-activated monocyte-derived DC. We collected supernatants from these assays to determine concentrations of the cytokines IL-6, IL-12p40 and TNF α induced under the varying culture conditions. In LPS-activated cultures, IL-10 neutralization resulted in potent restoration of levels of all three cytokines (Figure 4.6 B), again mimicking the effects we observed with TLR7/8 agonist R848. The effect is strikingly obvious, and the lack of statistical significance in this set of data is therefore most likely due to the small cohort of four independent experiments ($n = 4$).

As outlined in chapter 3, in comparison to activation with R848 or LPS, upon poly I:C stimulation, monocyte-derived DC produce considerably lower levels of cytokines. We nevertheless attempted to measure IL-6, TNF α and IL-12p40 levels in these assays,

but many values fell below the sensitivity threshold of our sandwich ELISAs, and these data are therefore not available.

Collectively, the assays employing specific neutralizing antibodies for selected cytokines have shown that while the mechanisms of suppression of monocyte-derived DC activation by both R848 and LPS are mediated primarily by IL-10, this factor does not appear to play a role when activation is induced by TLR3 agonist poly I:C. Although these findings are intriguing, due to the poor cytokine induction by poly I:C, CD86 provided the only reliable activation marker in our assays, and this limited our possibilities to further dissect the underlying mechanisms and factors in ascites underlying the suppression of activation by poly I:C in monocyte-derived DC. We therefore did not pursue this path in more detail, but focussed in our subsequent experiments further on TLR7/8-mediated DC activation and suppression thereof by ovarian carcinoma associated ascites.

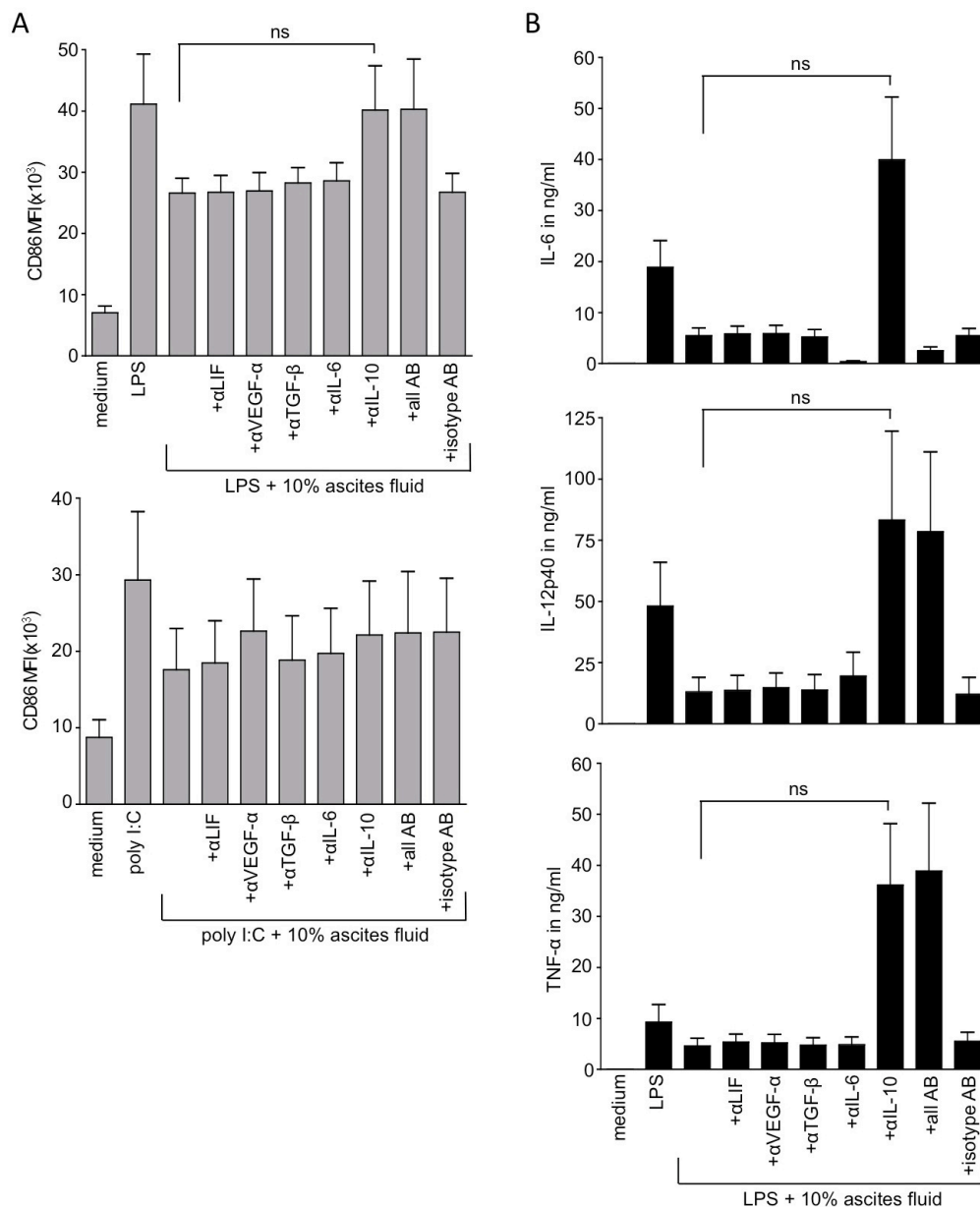


Figure 4.6 Neutralization of IL-10 in cell cultures alleviates the suppression of LPS- but not poly I:C-mediated monocyte-derived DC activation.

Monocyte derived DC were cultured in the presence of 1μg/ml LPS or 100μg/ml poly I:C, 10% ascites and neutralizing antibodies (5μg/ml) as indicated. Neutralizing antibodies were added to cultures concomitantly with TLR agonists and ascites, and were added alone or in combination as indicated. **(A)** CD86 expression levels were measured by flow-cytometry, and **(B)** cytokine levels were measured in cell culture supernatants by Flow Cytomix analysis (IL-6 and TNFα) or sandwich ELISA (IL-12p40). For LPS n=4 (4 independent experiments; monocyte-derived DC from 4 different healthy donors, cultured with one of two different ascites each) and for poly I:C n=3 (3 independent experiments; monocyte-derived DC from 3 different healthy donors, cultured with one of two different ascites each). One-way ANOVA (Friedman test with Dunn post test): ns = not significant. Please note that the quantification of IL-6 in samples containing IL-6 neutralizing antibody (αIL-6 and all AB) is compromised.

4.5 Ovarian carcinoma associated ascites impairs the T cell stimulatory capacity of monocyte-derived DC *in vitro*

DC are professional APC and their function as such is pivotal to the initiation of an anti-tumour immune response. They are required for the presentation of TAA to naïve CD4⁺ and CD8⁺ T lymphocytes which are thereby primed and expand to form populations of tumour specific CD4⁺ T_H and CD8⁺ cytotoxic T effector cells. Having observed impaired activation of monocyte-derived DC in presence of malignant ascites in our previous experiments, we explored the question whether such reduced activation further negatively influenced the T cell stimulatory capacity of DC and if so, whether impaired functionality equally appeared to be dependent on IL-10.

To address this, we conducted allogeneic MLR, using monocyte-derived DC from one healthy donor and stimulating them overnight with TLR7/8 agonist R848 in absence or presence of 10% of ovarian carcinoma associated ascites and/or neutralizing IL-10 antibody. After overnight culture, the DC were thoroughly washed to prevent carry-over of R848, ascites or α IL-10 neutralizing antibody and seeded onto fresh plates together with CFSE stained naïve CD4⁺ T cells at different DC : T cell ratios (1:25, 1:50, 1:100 and 1:200). After incubation over 6 days, cells were harvested and T cell proliferation was assessed by CFSE dilution as measured by flow cytometry (Figure 4.7). We observed an increased induction of T cell proliferation by DC pre-cultured in presence of R848 as compared to medium only. Importantly, where ascites was present in DC cultures, the T cell stimulatory capacity was reduced, and again restored beyond baseline levels (R848 only) upon IL-10 neutralization. These data were consistent for all experiments performed but did not reach statistical significance, most likely due to the limited number of assay repetitions performed ($n = 4$). Our observations in these experiments complimented the previous results and showed that reduced activation of monocyte-derived DC in presence of ovarian

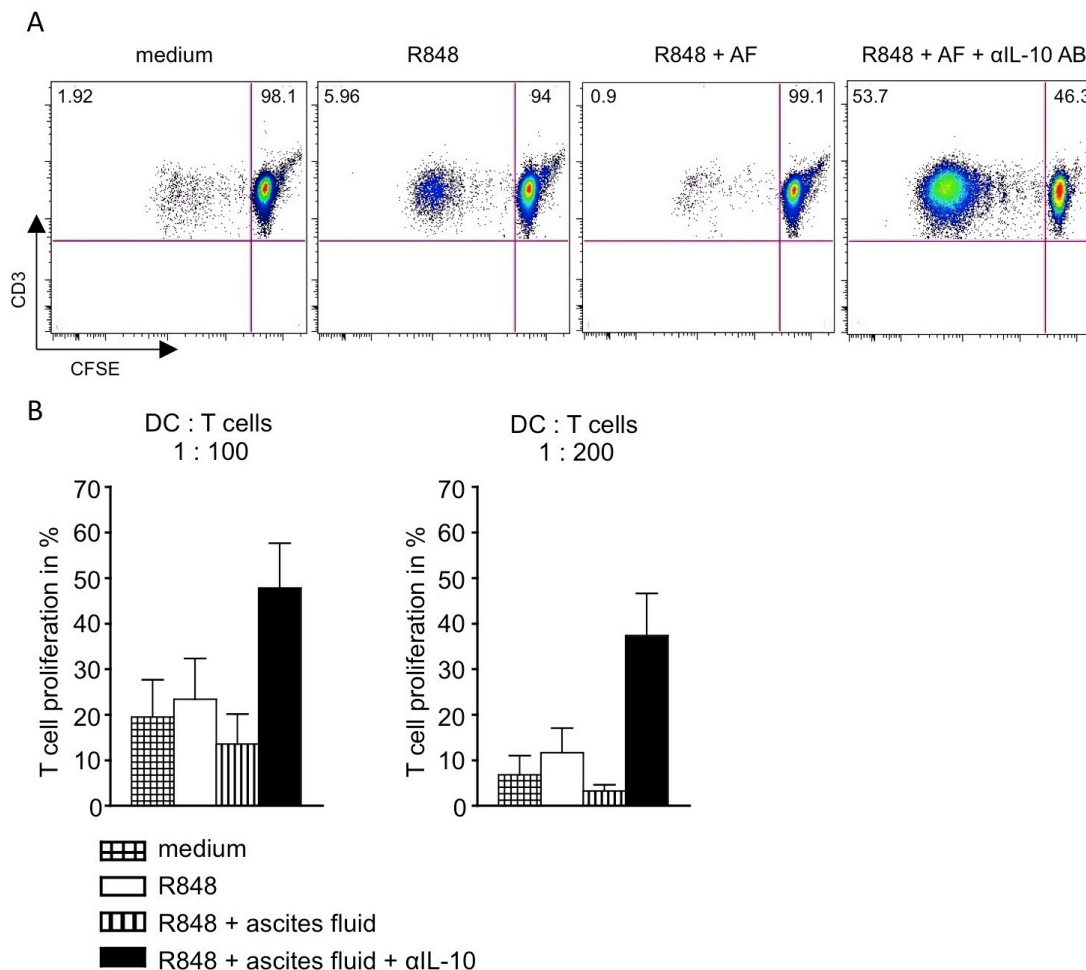


Figure 4.7 Ovarian carcinoma associated ascites impairs the T cell stimulatory capacity of monocyte-derived DC, but functionality can be restored by IL-10 neutralization.

For allogeneic MLR, monocyte-derived DC from healthy donors were cultured overnight in complete medium only, or stimulated with 3 µg/ml R848 in the presence or absence of 10% ascites (AF) and/or IL-10 neutralizing antibody (AB) (5 µg/ml) as indicated. Subsequently, such pre-treated DC were washed and co-cultured with CFSE-stained CD4⁺ naïve T cells from an other healthy donor at a 1:100 or 1:200 ratio for 6 days. Naïve T cells from the same donor were used consistently for all experiments. The proliferation of T cells was determined by CFSE dilution after 6 days. **(A)** A representative example of flow-cytometry analysis is shown (DC : T-cell ratio 1:200). After gating on CD3⁺ cells, proliferating and non-proliferated T lymphocytes are shown as assessed by CFSE dilution. **(B)** Cumulative data from 4 independent experiments showing proliferation in percent of CD3⁺ cells that have undergone at least one round of division. n=4 (4 independent experiments; monocyte-derived DC from 3 different healthy donors; DC from two donors were cultured with only one ascites sample, while the third donor's cells were cultured with ascites from two different ovarian carcinoma patients).

carcinoma associated ascites negatively influences the T cell stimulatory capacity of these APC, resulting in reduced T cell proliferation *in vitro*.

In addition to T cell proliferation, we also considered the possibility that DC pre-cultured in presence of ascites may alter the type of CD4⁺ T cell response induced. To address this, we performed intracellular staining of T cells for transcription factor FoxP3 as well as the cytokines IL-10, IL-17 and IFN γ after 6 days of MLR culture (Figure 4.8). We noticed a slight decrease in FoxP3⁺ cells upon activation with R848, but detected no alteration between R848 activated samples with or without ascites and/or α IL-10 antibody, with percentages of FoxP3⁺ cells ranging between 3.5 - 4.5% in these samples (Figure 4.8 bottom row). The percentage values of IFN γ ⁺ cells decreased slightly from 22% to 17% with R848 activation, and a slight increase from 17% to 21% was then noted with the addition of ascites (Figure 4.8 top row). The percentage of IFN γ ⁺ cells doubled where α IL-10 antibody was added to DC cultures, but since we saw no decrease of IFN γ induction in presence of ascites, this is most likely due to neutralization of autocrine IL-10. In all samples, only a very small percentage of T cells (<1%) produced IL-10 or IL-17.

The data from these MLR assays show that the influence of ovarian carcinoma associated ascites on monocyte-derived DC outlasts their immediate exposure to the fluid, and that not only their direct activation via TLR8 is reduced but equally their capacity to stimulate naïve T cells is partially impaired.

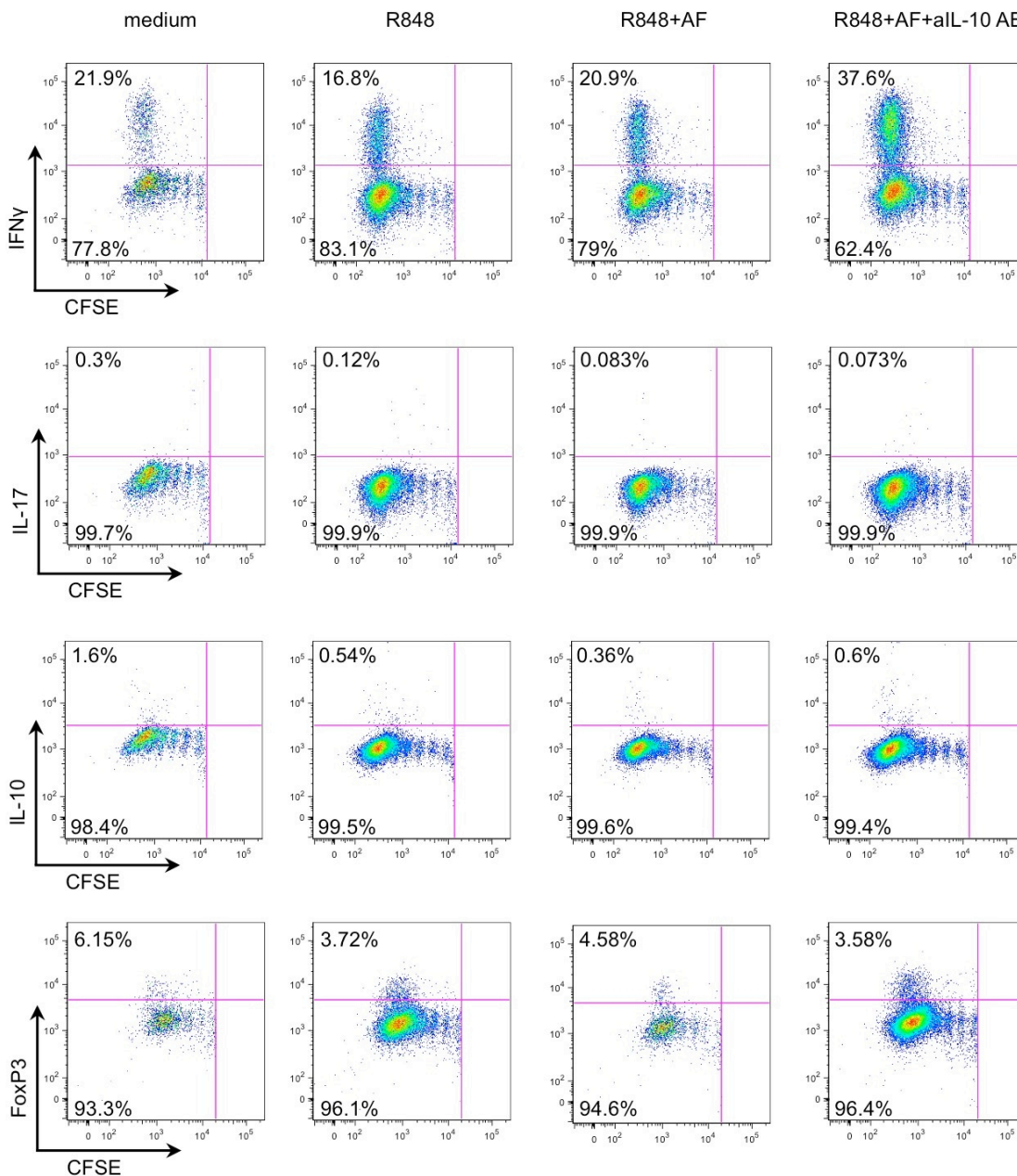


Figure 4.8 The CD4⁺ T cell response induced by monocyte-derived DC in MLR reactions is not altered towards a regulatory or T_H17 phenotype by ovarian carcinoma associated ascites.

For allogeneic MLR, monocyte-derived DC from healthy donors were cultured overnight in complete medium only, or stimulated with 3 μ g/ml R848 in the presence or absence of 10% ascites (AF) and/or IL-10 neutralizing antibody (AB) (5 μ g/ml) as indicated. Subsequently, such pre-treated DC were washed and co-cultured with CFSE-stained CD4⁺ naïve T cells from an other healthy donor at a 1:200 ratio for 6 days. The proliferation of T cells was determined by CFSE dilution after 6 days. After gating on CD3⁺ cells that have undergone at least one round of division, percentage values of proliferating cells producing the cytokines IFN γ , IL-10 or IL-17, or cells positive for transcription factor FoxP3 are shown. One representative example is shown of 2 independent experiments for IFN γ , IL-10 IL-17, and of 3 independent experiments for FoxP3.

4.6 Factors other than IL-10 in ovarian carcinoma associated ascites contribute to suppression of TLR-mediated monocyte-derived DC activation

Although our experimental results thus far pointed towards a pivotal role of IL-10 as mediator of suppression of monocyte-derived DC activation via TLR in presence of ascites from ovarian carcinoma patients, given the complex composition of this tumour environment, we could not exclude that other ascites-derived factors not yet addressed in our study were also contributing to the impairment of DC activation.

We therefore performed experiments to compare activation levels of monocyte-derived DC activated with R848 in presence or absence of ascites, having eliminated the influence of IL-10 by including specific IL-10 neutralizing antibody to both culture conditions. Levels of CD86, IL-6, IL-12p40 and TNF α were markedly lower in samples containing ascites, suggesting that factors other than IL-10 were impeding monocyte-derived DC activation in these samples (Figure 4.9 A). This difference only reached statistical significance for the surface marker CD86, however the trend was also consistent for all three cytokines. The relevance of these data is augmented when compared with control experiments using peritoneal fluid from patients with benign ovarian tumours (Figure 4.9 B). When IL-10 was neutralized in these cultures, we detected no difference between samples with or without peritoneal fluid, indicating that once IL-10 is eliminated as a driver of suppression, no further factors in benign peritoneal fluid impede TLR-mediated monocyte-derived DC activation.

Again, as seen previously (see chapter 3, Figure 3.7 B), benign peritoneal fluid failed to suppress TNF α production (Figure 4.9 B, bottom graph), which marked a difference to malignant ascites (see Figure 4.9 A bottom graph and chapter 3, Figure 3.6 B).

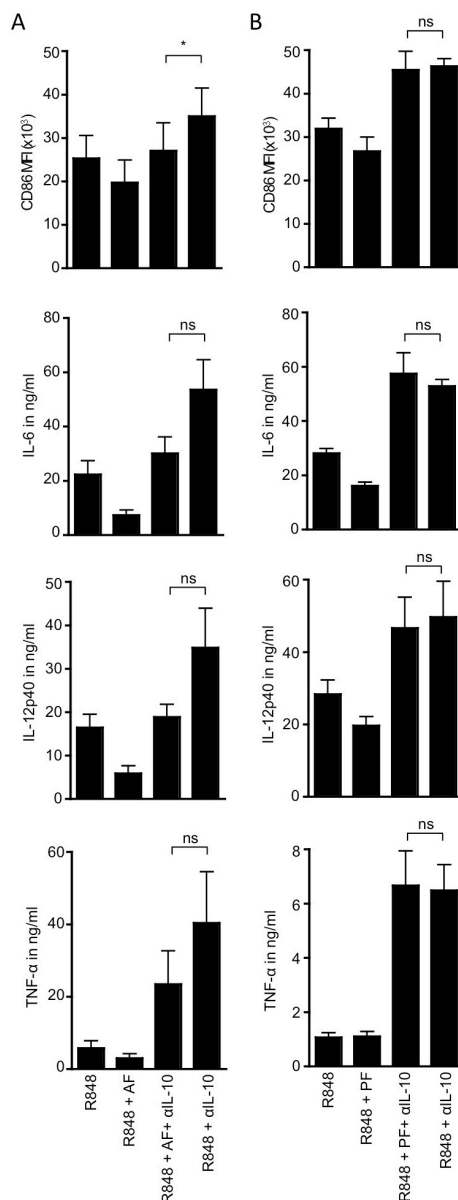


Figure 4.9 Factors other than IL-10 that impair TLR-mediated monocyte-derived DC activation are present in ovarian carcinoma ascites but not peritoneal fluid from patients with benign ovarian tumours.

Monocyte-derived DC were stimulated overnight with 3µg/ml R848 in the presence or absence of (A) 10% malignant ascites (AF) from OC patients or (B) 10% peritoneal fluid (PF) from benign ovarian tumours and IL-10 neutralizing antibody (5µg/ml) as indicated. CD86 expression levels were assessed by flow-cytometry, and cytokine levels were measured in cell culture supernatants by flow cytometry analysis (A: IL-6 and TNFα) or sandwich ELISA (A: IL-12p40 and B: TNFα, IL-6 and IL-12p40); for malignant samples n=6 (DC from five different healthy volunteers were cultured with ascites from 5 different ovarian carcinoma patients. Altogether, 6 independent experiments were performed. Cells from four volunteers were cultured with only one ascites sample each, while cells from the remaining donor were cultured with two different ascites samples.; for benign samples n=9 (DC from five different healthy volunteers were cultured with peritoneal fluid from 6 different patients. Altogether, 9 independent experiments were performed. Cells from three volunteers were cultured with only one ascites sample each, while cells from the remaining two donors were cultured with three different ascites samples each); One-way ANOVA (Friedman test with Dunn post test): * = p<0.05, ns = not significant.

We further recall that although TNF α was potently suppressed by ovarian carcinoma-associated ascites, no correlation between magnitude of suppression and IL-10 levels in ascites had been detected (see above, Figure 4.2 bottom right graph). All these observations taken together led us to hypothesize that one or several additional suppressive factors other than IL-10 are present in ascites and absent from benign peritoneal fluid, and that more specifically, these may contribute to and possibly correlate with the suppression of TNF α production by monocyte-derived DC upon R848 stimulation.

As reviewed in the introduction to this chapter, a large number of proteins with immunosuppressive properties have been reported to accumulate in ovarian carcinoma-associated ascites. The most promising candidates we considered included sCTLA-4, Arg-1 as well as binding immunoglobulin protein (BIP), but we could exclude the role of all these factors in our assays after several simple pilot experiments briefly outlined below.

sCTLA-4 was not detectable in any of our samples (data not shown) and was therefore swiftly dismissed. Arg-1 was abundant in some ovarian carcinoma ascites samples, but one of the three benign ovarian samples that were available to us at this time also displayed very high levels of Arg-1 (Figure 4.10 A). A lack of correlation of Arg-1 in malignant ascites samples with TNF α suppression (see above, Table 4.1) further made this protein an unlikely candidate. In collaboration with Dr. Valerie Corrigan, CMCBI, King's College London, BIP was measured in both malignant and benign fluid samples, with malignant samples showing higher levels of this immunosuppressive protein (Figure 4.10 B, this ELISA was performed and analyzed by Dr. Chris Hall). Following these encouraging results, in order to study in more detail the influence of BIP on R848-mediated activation with a focus on suppression of TNF α , we conducted experiments adding recombinant BIP to monocyte-derived DC cultures at a range of concentrations resembling those found in our ascites samples (recombinant BIP was provided by Dr. Corrigan). CD86 up-regulation remained unaffected by BIP, but

surprisingly, we observed enhanced levels of TNF α as well as IL-6 and IL-12p40 in cultures where BIP was added (Figure 4.10 C). As this was contradictory to our hypothesis, we did not further pursue this candidate protein as a possible contributor to suppression of TLR-mediated monocyte-derived DC activation.

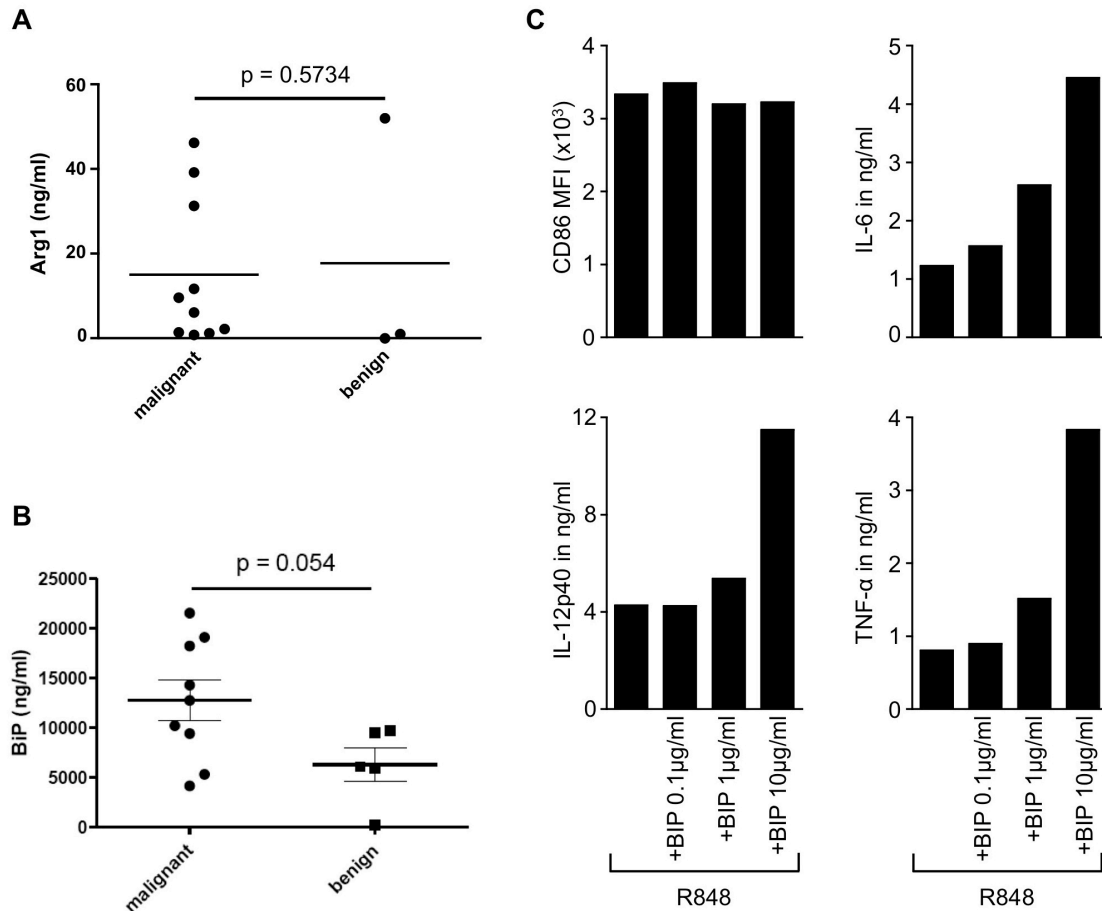


Figure 4.10 Arg-1 and BIP levels in malignant ascites versus benign peritoneal fluid samples and influence of recombinant BIP on R848-mediated DC activation.

(A) Arg-1 levels were measured in all malignant ascites and benign peritoneal fluid samples available to us at the given time in the study by sandwich ELISA. **(B)** BiP sandwich ELISA with all of our available malignant and benign samples was performed by Dr. Chris Hall (CMCBI, King's College London), who also analyzed these data. For both Arg-1 and BiP, Mann-Whitney U test was used for statistical analysis. **(C)** Monocyte-derived DC were stimulated overnight with 3 μ g/ml R848 with or without recombinant BIP at three different concentrations as indicated. CD86 expression was assessed by flow cytometry and cytokine levels in cell culture supernatants were measured by sandwich ELISA. This assay using recombinant BIP was performed one time only (n=1).

4.7 PGE₂ and IL-10 in ovarian carcinoma associated ascites have an additive effect on suppression of TLR-mediated TNF α induction

PGE₂ is a protein with multiple functions in the human body ranging from smooth muscle relaxation, vasodilative activity to a well documented, if complex and not yet fully understood role in the immune system. PGE₂ is a derivate of arachidonic acid (AA) belonging to the class of eicosanoids, and it exerts its functions via four different receptors EP1 – EP4 (Kalinski, 2012). Monocyte-derived DC express receptors EP2 and EP4 and PGE₂ has been described to modulate cytokine expression, including IL-10, IL-12p40 and TNF α in these and other immune cells (Kalinski et al., 1997; Laudanski et al., 2004; Hubbard et al., 2010; Poloso et al., 2013). Further, PGE₂ has previously been detected in ovarian carcinoma-associated ascites (Lavoue et al., 2012) and ovarian carcinoma cells show overexpression of cyclooxygenase-1 and -2 (COX-1 and COX-2, respectively), the key enzymes in the synthesis of eicosanoids including PGE₂. For these reasons, PGE₂ was a promising candidate and possible factor in ovarian carcinoma-associated ascites contributing to suppression of TLR-mediated activation of monocyte-derived DC.

Pilot experiments using recombinant PGE₂ in cell cultures yielded promising results, showing impaired R848-induced TNF α production in presence of PGE₂ (Figure 4.11). We tested three concentrations of recombinant PGE₂ in this assay, chosen in reference to a previous study which reported levels of PGE₂ in ovarian carcinoma ascites of up to 40 ng/ml (Lavoue et al., 2012). Similarly to TNF α , the levels of IL-6 and IL-12p40 were reduced where recombinant PGE₂ was added to cell cultures, but in contrast, CD86 up-regulation was enhanced by PGE₂. For all cytokines, the suppressive effect was dose dependent, with higher concentrations of PGE₂ inducing more prominent suppression. As a next step following these encouraging results, we neutralized PGE₂ in cell cultures containing ovarian carcinoma ascites, and

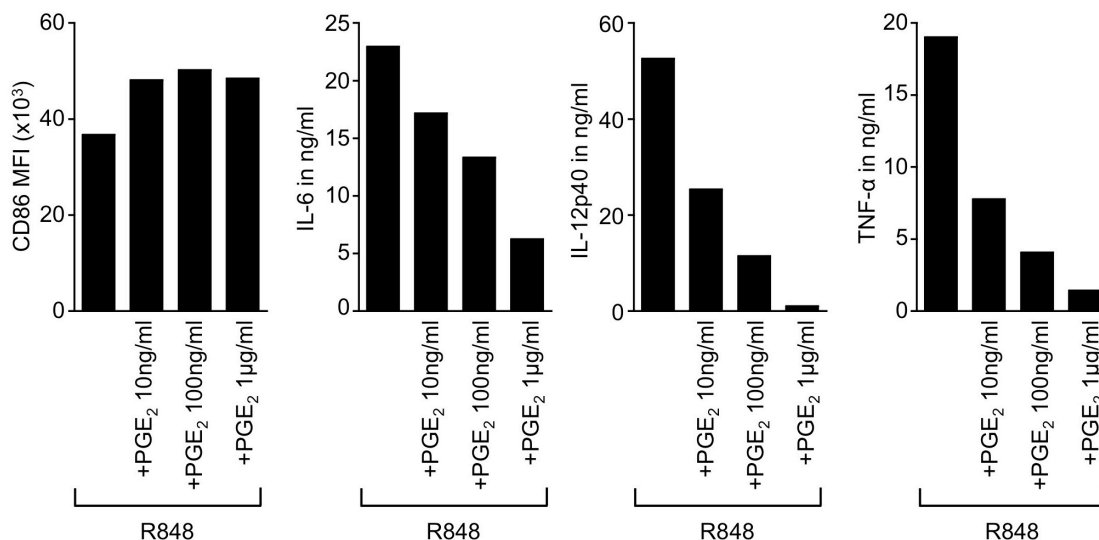


Figure 4.11 Impact of recombinant PGE₂ on R848-mediated monocyte-derived DC activation.

Monocyte-derived DC were stimulated overnight with 3μg/ml R848 with or without recombinant PGE₂ at three different concentrations as indicated. CD86 expression was assessed by flow cytometry and cytokine levels in cell culture supernatants were measured by sandwich ELISA. This assay using recombinant PGE₂ was performed one time only (n=1).

observed a partially restored TNFα production in samples where PGE₂ was blocked (Figure 4.12 A), while CD86, IL-6 and IL-12p40 levels remained unaffected (Figure 4.12 B). Concomitant neutralization of IL-10 and PGE₂ led to higher TNFα production than neutralization of IL-10 alone (Figure 4.12 C). While the neutralization of IL-10 was the major factor alleviating TNFα suppression, the contribution of PGE₂ was consistent, and albeit small, the increase in TNFα production upon additional PGE₂ neutralization was statistically significant.

To exclude the possibility that the observed effects of PGE₂ were caused by neutralization of autocrine PGE₂ produced by DC upon TLR activation, we neutralized PGE₂ in R848-activated cultures without ascites. We saw no effect of PGE₂ neutralization on TNFα levels in response to R848-mediated activation (Figure 4.12 D), which led us to believe that autocrine PGE₂ does not play a role in the suppression of TLR-induced TNFα production by monocyte-derived DC.

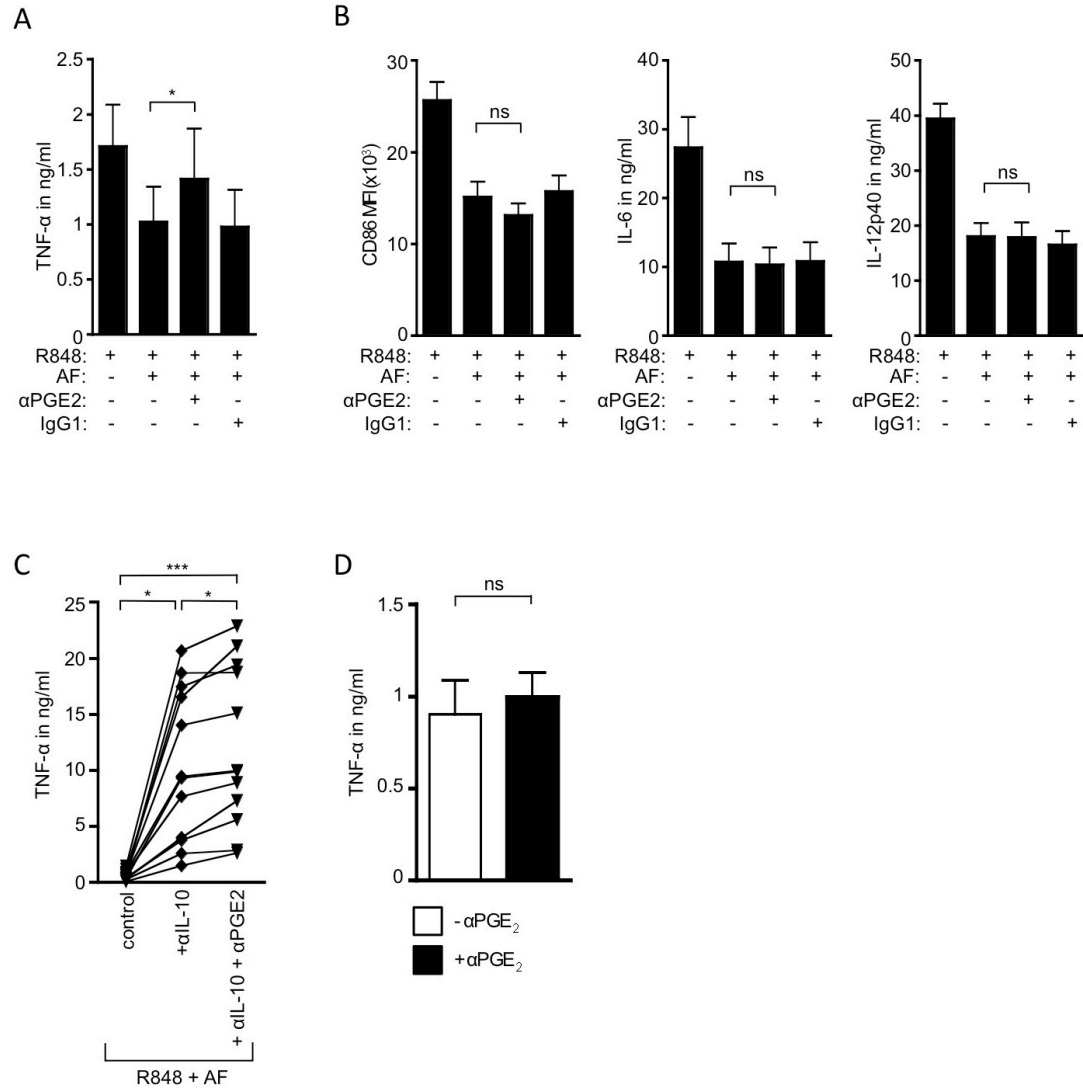


Figure 4.12 Neutralization of PGE₂ partially restores R848-induced TNF α production and enhances TNF α levels beyond those reached by IL-10 neutralization alone.

Monocyte-derived DC were stimulated overnight with 3 μ g/ml R848. **(A-B)** Malignant ascites and neutralizing antibodies (5 μ g/ml) against PGE₂ were added as indicated; n=11 (11 independent experiments; monocyte-derived DC from four different healthy volunteers were cultured with ascites from four different ovarian carcinoma patients. Altogether, 11 independent experiments were performed. Cells from three volunteers were cultured with three different ascites sample each, while cells from the remaining donor were cultured with only two different ascites samples. **(C)** Neutralizing antibodies against IL-10 and PGE₂ were added as indicated to cultures containing 3 μ g/ml R848 and 10% ascites; n=9 (DC from four different healthy volunteers were cultured with ascites from 5 different ovarian carcinoma patients. Altogether, 9 independent experiments were performed. Cells from three volunteers were cultured with two ascites samples each, while cells from the remaining two donors were cultured with either one or two different ascites samples, respectively. One-way ANOVA (Friedman test with Dunn post test): * = p<0.05; *** = p<0.001; ns = not significant; **(D)** Monocyte-derived DC were stimulated overnight with 3 μ g/ml R848. To exclude a role of autocrine PGE₂ on monocyte-derived DC activation, PGE₂-specific neutralizing antibody (5 μ g/ml) was added as indicated. n=3. Wilcoxon matched pairs test; ns = not significant. Cytokine levels in all cell culture supernatants were measured by sandwich ELISA.

To further confirm PGE₂ as a factor that distinguishes the malignant ovarian carcinoma environment from that of benign ovarian tumours, we measured the levels of PGE₂ in malignant ascites and benign peritoneal fluid samples used in our study (Figure 4.13). The levels of PGE₂ in malignant ascites vary between patients and while some samples were negative for this protein, most malignant ascites samples contained detectable levels of PGE₂. Interestingly however, all samples of peritoneal fluid from benign ovarian conditions showed very low or not detectable levels of PGE₂. Hence, our data suggest that PGE₂ is a factor absent from benign ovarian tumours but often present in the malignant OC environment that specifically impairs TLR-mediated TNF α induction by monocyte-derived DC.

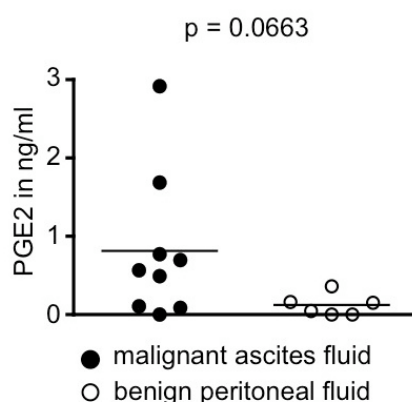


Figure 4.13 PGE₂ levels in ascites from ovarian carcinoma patients or in peritoneal fluid from patients suffering from benign tumours of the ovary.

PGE₂ levels in malignant ascites (n=9) and benign peritoneal fluid (n=6) were measured by sandwich ELISA. Mann-Whitney U test was used for statistical analysis.

4.8 TLR-mediated activation of primary myeloid DC in presence of ovarian carcinoma associated ascites

For reasons of practicability as outlined in the introduction to chapter 3, we conducted this study with the use of *in vitro* generated monocyte-derived DC. Work with primary human DC subsets is challenging due to the low numbers present in peripheral blood, typically the only material readily available from healthy donors. However, in the wider context of our study, especially when extrapolating our data to experimental and clinical settings and immunotherapeutic strategies exploring *in vivo* targeting strategies of primary DC subsets, it is of interest to investigate how our findings translate to myeloid DC, and whether ovarian carcinoma associated ascites impairs their activation by TLR agonists in similar fashion to monocyte-derived DC.

Human DC are divided into plasmacytoid and myeloid DC subsets which bear specific roles and functions as extensively described in chapter 1. We have decided to study the influence of ovarian carcinoma associated ascites on TLR-mediated activation of the myeloid DC 1 (mDC1) subset. While mDC2 are thought to be specialized in cross-presenting cell-associated antigens to CD8⁺ T cells and therefore of pivotal importance to anti-tumour immunity, this subpopulation is extremely sparse and sufficient numbers of these cells for conducting activation assays can't be obtained from peripheral blood. We therefore decided to focus our efforts on the mDC1 subset, which can be distinguished within PBMC with the help of the phenotypic marker BDCA-1 (CD1c) (Dzionek et al., 2000). Kits for DC isolation from peripheral blood are commercially available, and to ensure we obtain very high purity of mDC1, we combined the use of a panDC negative selection enrichment kit with fluorescent-activated cell sorting. BDCA-1 is also expressed by a small number of B lymphocytes (Small et al., 1987), and a concomitant staining with the B cell marker CD19 is therefore necessary to ensure accurate recognition of the desired mDC1 cell population. An analysis of the sorted cells including gating strategy was provided by

our departmental flow cytometry facility, and is shown in Figure 4.14 A. The starting population of PBMC was 4.2×10^8 which was obtained from a buffy coat from the National Blood Service. However, 4.2×10^8 constituted only 30% of all PBMC isolated from this buffy coat sample, with the remaining cells used for other experiments. From 4.2×10^8 PBMC, using a negative selection kit, we isolated 7.6×10^6 panDC (containing pDC, mDC1 and mDC2). Staining of panDC with BDCA1 and CD19 specific antibodies was followed by FACS sorting, and the obtained cell population of pure BDCA1⁺CD19⁻ mDC1 was approximately 1×10^6 (13% of panDC; 0.24% of starting PBMC). Since mDC1 are an abundant DC subpopulation and the FACS sort gating analysis indicates that 48.2% of all sorted cells fell within the BDCA1⁺CD19⁻ gate (Figure 4.14 A), the yield of 13% revealed that many cells were lost in the process of FACS sorting. Although the obtained number of cells (1×10^6 mDC1) was rather low, it nevertheless allowed us to perform an experiment with several culture conditions as tested previously with monocyte-derived DC. mDC1 express TLR8 (Jarrossay et al., 2001), and R848 was therefore a suitable agonist to induce mDC1 activation. While we observed an increase in CD86 levels upon culture with R848, interestingly, we did not see a suppression but rather a slight enhancement of CD86 up-regulation in presence of 10% ovarian carcinoma associated ascites (Figure 4.14 B). Neutralization of IL-10 with a specific antibody in cultures containing ascites did not alter the levels of CD86 considerably. In two of three experiments performed, we also determined the levels of IL-6 and TNF α in cell culture supernatants. We observed a production of considerable levels of both cytokines upon R848 activation, no suppression of IL-6 and a slight enhancement of TNF α levels in presence of ascites (Figure 4.14 B). IL-10 neutralization induced levels of both IL-6 and TNF α beyond those of samples cultured with R848 only, showing that autocrine IL-10 is affecting mDC1 activation.

Although these findings only constitute pilot experiments, it would appear that primary mDC1 may not be as susceptible to suppression of TLR8-mediated activation by

ovarian carcinoma ascites as monocyte-derived DC. However, due to the extremely limited data available, these results cannot yet be interpreted conclusively. These experiments were performed primarily as a proof of concept to establish protocols for purification of a primary human mDC subset, which we have achieved successfully. While the numbers that could be obtained limit the feasibility of assays that can be performed with these cells, by using higher starting numbers of PBMC from whole buffy coats from the National Blood Service, the format could be scaled up and specific questions regarding influence of ovarian carcinoma ascites on mDC activation could be addressed using these protocols. Design of future experiments to this effect is currently under consideration in our laboratory.

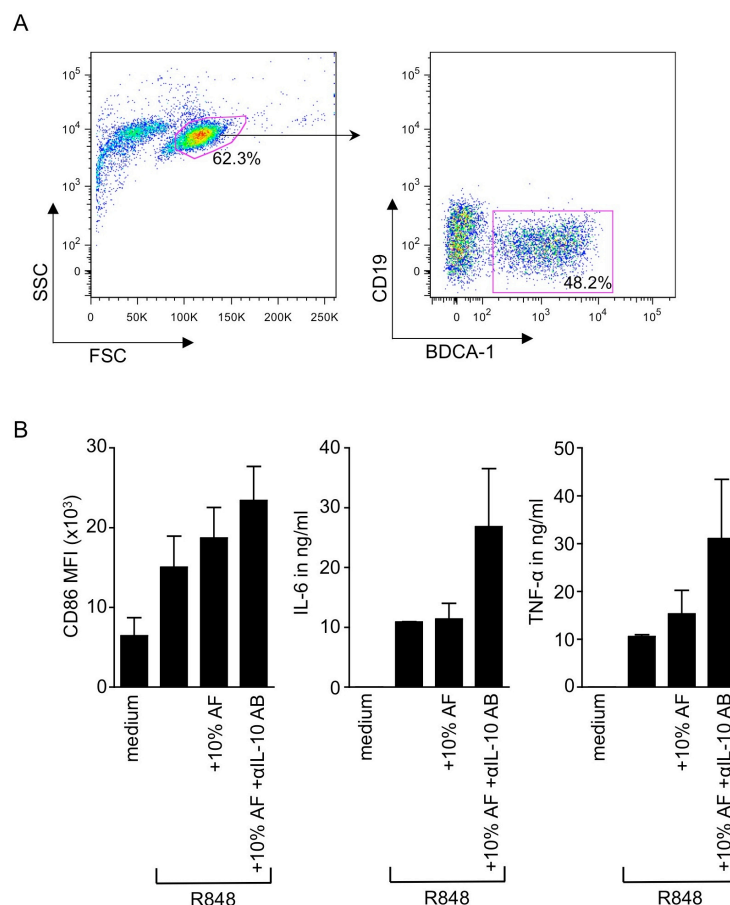


Figure 4.14 Influence of ovarian carcinoma associated ascites on R848-mediated activation of primary mDC1.

(A) Pan-DC were obtained by magnetic negative selection and stained with antibodies for CD19 and mDC1 marker BDCA-1 before FACS sorting. A representative example of the FACS sort gating is shown, with mDC1 defined as BDCA1⁺CD19⁻ population. **(B)** mDC1 were stimulated overnight with 3 μ g/ml R848 in the presence or absence of 10% malignant ascites from OC patients and IL-10 neutralizing antibody (5 μ g/ml) as indicated. CD86 expression levels were assessed by flow-cytometry, and cytokine levels were measured in cell culture supernatants by FlowCytomix analysis. For CD86 $n=3$ (3 independent experiments; mDC from 3 different healthy donors, cultured with 1 ascites); cytokines were measured in two of three experiments; $n=2$

Discussion

Data presented in this chapter inform about the composition of soluble factors within the ovarian carcinoma tumour environment, revealing the presence of an array of known immunosuppressive proteins. The contribution of these factors to the impairment of TLR-mediated activation of monocyte-derived DC was addressed with the use of specific neutralizing antibodies. Identifying proteins that suppress DC activation is an important step towards gaining an understanding of the ovarian carcinoma environment and the obstacles it may pose to immunotherapeutic strategies relying on activation of DC in situ.

Using selective neutralizing antibodies for an array of factors with known immunosuppressive properties, we attempted to elucidate their individual roles in the impairment of TLR-mediated activation of monocyte-derived DC. Our data have shown that neutralization of VEGF α , LIF, TGF β and IL-6 did not alleviate the suppressive effect of ovarian carcinoma associated ascites on monocyte-derived DC, and these factors therefore do not contribute to the impairment of DC activation in our study. These findings were perhaps somewhat surprising, because factors such as TGF β and VEGF α have previously been shown to negatively effect DC activation (Gabrilovich et al., 1996;Wallet et al., 2005). It is feasible that the tested proteins exert suppressive effects on DC only within a certain concentration range, and that amounts present in ascites during our cultures were not within this spectrum. Equally, these factors might bind to several receptors which may regulate DC activation in different ways. To elucidate all these possibilites, experiments with recombinant proteins at different concentrations, and use of specific receptor agonists and antagonists would be necessary, but this was not within the scope of our study.

It is important to note that the biological activity of the used neutralizing antibodies had not been tested in our study. Such experiments would complement this work, as they would allow for a more robust interpretation of the data obtained. Only with proof

of the capacity of the used antibodies to effectively neutralize the desired factors *in vitro* can one draw the definite conclusion that a particular agent does not play a role in an experimental system. The neutralizing ability of antibodies can be tested with the use of recombinant proteins, the *in vitro* neutralization of which can be verified either by simple quantitative measurement such as ELISA, or functional assays using responder cell lines. Appropriate experiments to verify the functionality of the used antibodies against VEGF α , LIF, TGF β and IL-6 are currently under consideration in our laboratory. Notably, with regards to our study, the use of cytokine-receptor blockers as an alternative to neutralizing antibodies represents an attractive option to verify the obtained data.

With the use of the selective neutralizing antibodies used to date, we could identify IL-10 and PGE₂ as two factors impairing TLR-mediated activation of monocyte-derived DC in distinct yet complementary ways. Our experimental data have shown that selective neutralization of IL-10 alleviated the suppression of monocyte-derived DC activation by TLR agonists potently and consistently, pointing towards a central role of IL-10 as driver of the observed suppression.

Interestingly, upon IL-10 blockade, the up-regulation of the surface marker CD86 and the production of IL-6 and TNF α were restored beyond the levels achieved in cells which were activated with R848 in absence of ascites. This observation was in line with the well documented IL-10 production by monocyte-derived DC upon TLR stimulation (Chang et al., 2000; Schreiber et al., 2010), and has shown that autocrine IL-10 impairs the cells' own activation and its neutralization can therefore further enhance DC activation. Notably, our preliminary and very limited data using primary mDC indicated that ascites-derived factors including IL-10 may not have a suppressive influence on R848-mediated activation of this primary DC subset. However, mDC are also known to produce IL-10 upon TLR activation (Jarrossay et al., 2001), and similarly to our assays with monocyte-derived DC, neutralization of IL-10 in mDC1 cultures enhanced activation levels beyond those achieved with TLR stimulation alone,

pointing also here towards a role of autocrine IL-10. Our attempts to address the impact of ascites-derived versus autocrine IL-10 on DC activation in our experiments, and the implications thereof, will be discussed in detail in chapter 5.

IL-10 is a much studied immunosuppressive protein with a documented role in ovarian carcinoma. In previous clinical studies, IL-10 was detected in ascites from ovarian carcinoma patients, and IL-10 serum levels are elevated in women suffering from the disease when compared to others with benign ovarian tumours or healthy controls not affected by any ovarian conditions (Gotlieb et al., 1992; Santin et al., 2001; Mustea et al., 2006; Yigit et al., 2011a).

Macrophages and T reg are considered an important source of IL-10 in the tumour microenvironment (Yigit et al., 2010; Wang et al., 2011). T reg are attracted to the malignant tissue via chemotactic signals such as CCL22, a factor produced by tumour cells and local leukocyte subsets, and the infiltration with T reg bears negative prognostic significance (Curiel et al., 2004). Similarly, monocytes are recruited to the tumour environment by chemotactic factors such as MCP-1 which is produced by ovarian carcinoma cells and can be detected in tumour tissue as well as ascites from ovarian carcinoma patients (Negus et al., 1995). Other factors which have been implicated to play a role in attracting monocytes to the tumour environment include macrophage colony stimulating factor (M-CSF), chemokine receptor 2 (CCR2) and VEGF α (Bingle et al., 2002). Once monocytes infiltrate the tumour tissue, their differentiation into macrophages is influenced by factors in situ, many of which favour their polarization into an M2 phenotype. Factors that lead to the induction of M2 macrophages include LIF, IL-4 and IL-10 (Sica et al., 2006; Duluc et al., 2007), all of which can be detected in ovarian carcinoma microenvironment (Yigit et al., 2011b). The M2 macrophage phenotype is characterized by an IL-10^{hi} and IL-12^{lo} cytokine secretion profile, and this cell population is specifically referred to as TAM. Apart from IL-10, other factors secreted by TAM shape the composition of the local tissue towards a pro-tumorigenic, immunosuppressive state by recruiting specific cell types

such as T reg (Curiel et al., 2004) and promoting tumour cell proliferation and neo-vascularization (Sica et al., 2006). The impact of TAM on the tumour environment is manifold and can influence disease progression. The infiltration of solid tumours with TAM has been repeatedly suggested to be of prognostic significance, with high TAM infiltration being associated with poorer clinical outcomes in breast, prostate and cervix cancer and others (Bingle et al., 2002). Although TAM are present in the ovarian carcinoma environment, the studies investigating their prognostic value in this disease have thus far been inconclusive (Bingle et al., 2002; Zhang et al., 2012). A study by Wan et al. describes in the available abstract an association of TAM infiltration in ovarian carcinoma with poor prognosis, but the complete article is not available in english and it is therefore not possible to verify the authors' conclusions (Wan et al., 2009).

IL-10 is being debated to bear prognostic value in ovarian carcinoma, since IL-10 levels correlate negatively with the differentiation grade of the malignant tumour, i.e. patients with poorly differentiated ovarian carcinoma present with higher levels of IL-10 in both ascites and serum (Mustea et al., 2009). Apart from TAM, which are potent producers of IL-10 and an important source of this cytokine in the ovarian carcinoma environment, the ovarian carcinoma epithelium itself also produces considerable amounts of IL-10. Although this has not been specifically addressed in the study by Mustea et al. or any other studies to date, it would be interesting to explore whether there is, in fact, a direct link between IL-10 production by cancerous epithelium and its grade of differentiation. In any case, the contribution of the cells of tumour mass to IL-10 production in ovarian carcinoma patients is considerable, and this is reflected by the fact that IL-10 serum levels drop significantly after de-bulking surgery (Mustea et al., 2009).

In our study, selective neutralization of IL-10 alleviated suppressive effects of ovarian carcinoma associated ascites, largely restoring TLR-mediated monocyte-derived DC activation. We could further show that the effects of malignant ascites on DC extended

beyond their activation, impairing the cells' T cell stimulatory capacity. However, once DC activation was recovered by IL-10 blockade, the ability of monocyte-derived DC to induce T cell proliferation equally returned. In consideration of this data, reducing levels of IL-10 in the ovarian carcinoma environment could potentially be a desirable strategy to enhance immunotherapeutic concepts, especially those intending to activate DC in situ. The decline in local and systemic levels of IL-10 as observed after de-bulking surgery presents an elegant window of opportunity which should be considered when designing timeframes for DC vaccination trials.

Due to its many immunoregulatory functions in the body, a systemic blockade of IL-10 bears significant risk of uncontrolled immune activation. The importance of this cytokine in preventing chronic inflammation was demonstrated in murine IL-10 knock-out (KO) models, which have shown that in absence of IL-10, mice develop chronic enterocolitis due to a failure to control an immune response to physiological enteric antigens. When challenged with pathogens, IL-10 KO mice mounted acute immune responses of significantly higher magnitude and duration than wildtype mice (Kuhn et al., 1993; Rennick et al., 1995). Despite these substantial consequences of IL-10 absence, in a small clinical study in patients suffering from systemic lupus erythematosus (SLE), selective IL-10 blocking antibodies were administered systemically, and this was surprisingly well tolerated (Llorente et al., 2000). It is important to note, however, that these patients were concomitantly receiving glucocorticoids, methotrexate and/or other immunosuppressive agents in line with their regular treatment regime for SLE, and side effects of excessive immune activation may therefore have been masked. Systemic IL-10 blockade therefore remains a precarious strategy which should be approached with caution, and clinical trials in larger cohorts may not be feasible. However, due to the limitation of ovarian carcinoma metastases to the peritoneal cavity even at late clinical stages, an intraperitoneal administration of selective IL-10 blocking agents may present an attractive option to lower local IL-10 levels with the aim of enhancing

immunotherapeutic strategies *in situ*. It may be worthwhile exploring this rationale in *in vivo* models of murine ovarian carcinoma where IL-10 signaling has also been shown to be of central importance to the regulation of immune responses within the tumour environment (Hart et al., 2011).

While IL-10 was the main factor impairing TLR-mediated activation of monocyte-derived DC in our study, we observed a residual suppression of activation upon IL-10 blockade in presence of malignant ascites as compared to samples without ascites. This suggested that at least one additional factor suppressing DC activation other than IL-10 was present in ovarian carcinoma ascites. Interestingly, in an identical experimental setup using benign peritoneal fluid, we saw no further suppression exerted by the fluid once the influence of IL-10 was neutralized. These findings pointed towards the existence of one or several soluble factors exclusively present in the malignant ovarian tumour environment and absent from benign conditions. A further indication that certain components distinguish the malignant environment from the benign was the observation (presented and discussed in chapter 3) that TLR-mediated TNF α induction remained unaffected by benign peritoneal fluid, but was potently suppressed in presence of malignant ascites. Additionally, while we detected a positive correlation between IL-10 levels in malignant ascites samples and the magnitude of suppression of the cytokines IL-6 and IL-12 as well as the surface marker CD86, TNF α suppression showed no such correlation with IL-10 levels. This led us to believe that while IL-10 was of central importance to the suppression of IL-6, IL-12 and CD86, TNF α suppression may additionally be driven by one or more further factors which are only present in malignant ascites.

Further experiments with neutralizing antibodies revealed that PGE₂ selectively impairs TLR-mediated TNF α induction while leaving the other activation markers unaffected. PGE₂ is an inflammatory mediator with known immunosuppressive function and has been shown to be crucial for DC migration and to influence cytokine release in response to TLR activation (Legler et al., 2006; Kalinski, 2012; Poloso et al.,

2013). The study by Legler et al. suggests that varying concentrations of PGE₂ may exert opposing influence on cytokine production by monocyte-derived DC upon TLR stimulation, and that this is in part due to differential use of the two functionally expressed PGE₂ receptors on DC, EP2 and EP4. In our study, relatively low concentrations of PGE₂ (≤ 0.75 ng/ml) were measured in ascites samples, and it is feasible that while this concentration may have been sufficient to suppress TNF α , higher levels may be necessary to effect other activation parameters. This speculation is supported by the fact that in experiments using recombinant PGE₂ at considerably higher levels (≥ 10 ng/ml) than found in ascites, we did indeed detect a suppression of both IL-6, IL-12 as well as CD86. Experiments using a wide spectrum of concentrations of recombinant PGE₂ and selective PGE₂ receptor blockers would be necessary to elucidate in depth the precise mechanisms by which PGE₂ acts upon DC activation.

The selectivity of PGE₂-dependent suppression of TNF α in our study is remarkable. Evidence from a study in trauma patients suggests that TNF α induction in monocytes may be regulated by PGE₂, though the precise mechanism is likely to be very complex and not entirely understood (Laudanski et al., 2004). Similarly, it was shown in rodents that PGE₂ can suppress the release of TNF α from macrophages after TLR-stimulation (Renz et al., 1988; Hubbard et al., 2010).

The notion that PGE₂ is a factor exclusive to the malignant tumour environment where it selectively suppresses TLR-mediated TNF α induction was further supported by the fact that while we detected a range of different PGE₂ concentrations in the malignant ascites samples in our study, peritoneal fluid from patients with benign ovarian tumours was devoid of this protein. Reports describing PGE₂ as a factor associated with the malignant tumour environment have emerged in the recent past (Lavoue et al., 2012). PGE₂ contributes to the establishment and maintenance of an immunosuppressive microenvironment by promoting development of myeloid-derived suppressor cells (MDSC) and favouring the polarization of recruited monocytes into

TAM (Heusinkveld et al., 2011; Obermajer et al., 2012). As discussed above, TAM are major contributors of IL-10 to the tumour environment in ovarian carcinoma, and PGE₂ may therefore, in fact, indirectly enhance IL-10 levels via TAM induction. The major sources of PGE₂ in the course of an immune response are epithelial cells, fibroblasts and leukocytes infiltrating the site of inflammation (Kalinski, 2012) and these cells can equally be found within tumours. Compared to normal ovarian tissue and benign tumours, cancerous tissue in malignant ovarian tumours shows overexpression of cyclooxygenase-1 and -2 (COX-1 and COX-2, respectively), the key enzymes of PGE₂ synthesis (Rask et al., 2006). Inhibitors of COX-1 and COX-2 are a widely used class of drugs referred to as non-steroidal anti-inflammatory drugs (NSAID) and are regarded as potentially powerful drugs for treatment of malignant conditions (Menter et al., 2010). NSAID block the synthesis of eicosanoids including PGE₂ and are applied in a variety of medical conditions such as rheumatoid diseases. The use of COX inhibitors in cancer therapy and prevention is currently being explored and interestingly, COX inhibitors have previously been successfully used in murine experimental models of breast and lung tumours to enhance DC-based vaccines (Haas et al., 2006; Hahn et al., 2006). In light of our findings, the application of COX inhibitors may equally be an attractive measure to boost the efficacy of DC vaccines in ovarian carcinoma.

It is important to note that PGE₂ was not detected in all malignant ascites samples in our study, indicating that there may be two patient groups for which PGE₂ either does or does not play a role in the tumour microenvironment. Investigating whether presence of PGE₂ influences patient prognosis, correlates with other parameters of disease progression or affects susceptibility to treatment with COX inhibitors and/or immunotherapeutic intervention strategies would be of great interest. However, such hypotheses exceed the scope of our study, and much larger cohorts will be necessary to address these questions.

Chapter 5

Contribution of ascites-derived versus autocrine IL-10 and PGE₂ to suppression of TLR-mediated monocyte-derived DC activation

Introduction

As extensively shown and discussed in the previous chapters, stimulation of monocyte-derived DC with TLR agonists induces their activation, which is characterized by up-regulation of specific surface markers and production of pro-inflammatory cytokines. Previous reports have documented that upon TLR stimulation, DC equally produce the immunoregulatory protein IL-10 (Chang et al., 2000; Jarrossay et al., 2001), and this was also confirmed in our study (chapter 3, Figure 3.6 A). Concomitant induction of the immunoregulatory factor IL-10 under inflammatory conditions is an important protective mechanism of the immune system, serving to prevent excessive and uncontrolled immune activation. This protein can act in autocrine and paracrine manner, affecting both the producing cells as well as surrounding cells and tissues. While these processes take place during inflammatory responses *in vivo*, they equally bear significance in experimental *in vitro* systems, where factors secreted by cells in culture inevitably influence the culture conditions.

While studying which factors within ovarian carcinoma associated ascites impaired TLR-mediated activation of monocyte-derived DC in our assays, we selectively neutralized various immunosuppressive factors in cell cultures using specific antibodies. Our data have shown that neutralization of IL-10 leads to a potent restoration of DC activation in presence of ascites, pointing towards a central suppressive role of this cytokine in our experiments. However, the activation levels of

DC upon TLR stimulation in presence of IL-10 neutralizing antibody were considerably higher than those induced by TLR agonists alone. This observation revealed that autocrine IL-10 produced by monocyte-derived DC in cell cultures was contributing to the observed suppression, and prompted us to explore the relative importance of ascites-derived versus autocrine IL-10 in our experimental system.

We also identified PGE₂ as a factor present in ovarian carcinoma associated ascites which selectively impaired TNF α induction upon R848 stimulation. Previous reports indicate that different leukocyte populations produce PGE₂ in the course of an immune response (Kalinski, 2012). However, our data, albeit limited, did not necessarily suggest that production of PGE₂ by monocyte-derived DC played a role in our experiments (chapter 4, Figure 4.12 D). Our primary focus was therefore on the understanding of the roles of autocrine and ascites-derived IL-10, and in order to address this question, we designed an approach to selectively eliminate IL-10 from ascites samples used in our activation assays, while leaving autocrine IL-10 unaffected. To achieve this, we depleted IL-10 from ascites and used such depleted fluid in cell cultures without further addition of IL-10 neutralizing antibodies. Our endeavours to perform these experiments, their outcome and the difficulties we encountered along the way are outlined in detail in this chapter.

For a better understanding of the challenges we faced, it is of importance to emphasize that ovarian carcinoma associated ascites is a highly variable biological specimen containing an array of proteins, only a fraction of which we have quantified and addressed in our study. The pathophysiology of ascites formation has been outlined in chapter 1, and in malignant conditions that lead to ascites such as ovarian carcinoma, the fluid is particularly rich in proteins. The serum-ascites albumin gradient (SAAG) is a clinical parameter and diagnostic tool commonly used to distinguish between underlying conditions that lead to ascites formation (Chung and Kozuch, 2008):

$$\text{SAAG} = (\text{serum albumin concentration in g/dL}) - (\text{ascites albumin concentration in g/dL})$$

Albumin is the most abundant protein in human serum, its typical concentration ranging between 3.5 – 5.0 g/dL. Since ascites is essentially a filtrate of serum, albumin is also present in ascites, but its levels vary depending on the pathophysiological cause that leads to ascites formation in a particular case. In peritoneal carcinomatosis, the reasons for retention of liquid in the peritoneal cavity include local lymph vessel obstruction by tumour cells, hormonal imbalances and others. The hydrostatic and osmotic pressure between the circulation and the extravascular compartment remain unchanged or are only slightly altered, leading to an albumin concentration in ascites similar to or only a little lower than in serum. This results in a SAAG of typically <1.1 g/dL (Chung and Kozuch, 2008).

In contrast, if ascites is caused by high portal venous pressure, for example due to heart failure or liver cirrhosis, the hydrostatic pressure in the peritoneal venous circulation causes significantly more fluid to leave the blood vessels than is reabsorbed by osmotic pressure. Large amounts of protein-poor filtrate are accumulated in the peritoneal cavity while albumin is retained within the circulation. This leads to a relative dilution of extravascular protein leading to a considerably lower concentration of albumin in ascites than in serum. Consequently, in pathologies associated with high portal pressure, the SAAG reaches >1.1 g/dL (Kuiper et al., 2007).

The typically high concentration of albumin in ovarian carcinoma associated ascites as reflected in the SAAG values in this disease can be extrapolated to other serum proteins such as immunoglobulins, but equally to a plethora of factors not associated with immunological processes, making ascites abundant with soluble proteins (Gortzak-Uzan et al., 2008). Regardless of whether particular ascites constituents have immunological function and/or direct effect on read-out parameters monitored in our experiments, when attempting depletion of a specific protein from ascites, all components of this material have to be taken into account and, as we have learned, reckoned with as potential agents that may interfere with the experimental design.

5.1 Depletion of IL-10 from ovarian carcinoma associated ascites using α IL-10 antibody and Protein G agarose beads

The experimental design of the first approach we took to deplete IL-10 from ascites in order to study the contribution of autocrine versus ascites-derived IL-10 is schematically illustrated in Figure 5.1. The rationale comprised addition of the previously used IL-10 specific neutralizing antibody to pure ascites to enable IL-10 in ascites to bind to this antibody, forming antibody-antigen complexes. Subsequently, we intended to remove such complexes by centrifugation after addition of agarose beads coated with Protein G, an immunoglobulin binding protein with a high affinity to the Fc and Fab regions of antibodies. The affinity of Protein G varies considerably for antibodies of different isotype and host species. Its very high affinity for mouse IgG2b antibody, the precise isotype of the IL-10 neutralizing antibody used for this depletion, made Protein G a suitable candidate for these experiments.

In our initial pilot experiment, we incubated two malignant ascites samples from two ovarian carcinoma patients with IL-10 neutralizing antibody at two different concentrations of 5 μ g/ml (identical to the concentration previously used in cell culture) and 20 μ g/ml. The depletion was verified by measurement of IL-10 in the depleted samples, and indeed, IL-10 was not detectable in the samples depleted with the antibody concentration of 20 μ g/ml (data not shown). We therefore considered this depletion to be successful, and conducted several monocyte-derived DC activation assays with these samples. We further depleted two more samples of IL-10 with this method, using the antibody concentration of 20 μ g/ml. In the activation assays conducted with depleted ascites, we were interested to compare levels of activation induced by R848 in presence of non-depleted ascites with addition of IL-10 neutralizing antibody versus IL-10 depleted ascites from the same patient. Our results showed very similar levels of CD86, IL-6, IL-12p40 and TNF α induced under both culture conditions (Figure 5.2 A-B). These data could be interpreted as indication of

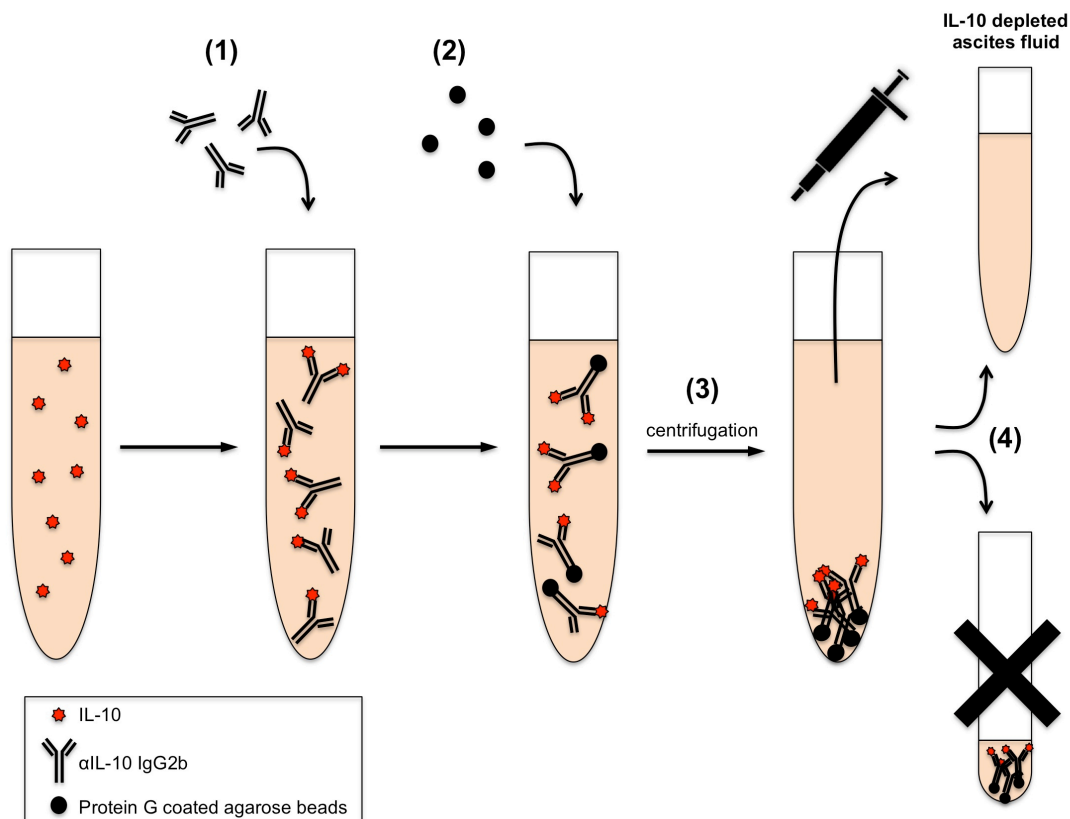


Figure 5.1 Schematic depiction of rationale behind the experimental approach using α IL-10 neutralizing antibody and Protein G coated agarose beads for depletion of IL-10 from ascites.

- (1) Neutralizing α IL-10 IgG2b antibody is added to ascites at a concentration of $20\mu\text{g/ml}$, binding all IL-10 present in ascites.
- (2) Protein G coated agarose beads are added, binding to the antibody-antigen complexes with high affinity.
- (3) Centrifugation creates a pellet of agarose beads with bound antibody-antigen complexes; the pellet is clearly visible at the bottom of the centrifuge tube.
- (4) The supernatant of depleted ascites is transferred into a fresh centrifuge tube and used in experiments as depleted, IL-10-free ascites. The pellet containing agarose beads and antibody-antigen complexes is discarded.

autocrine IL-10 playing a negligible role in the suppression of TLR-mediated activation, because in the depleted samples, such autocrine IL-10 was still present but not neutralized, and yet the DC activation levels were comparable to samples where autocrine IL-10 was blocked.

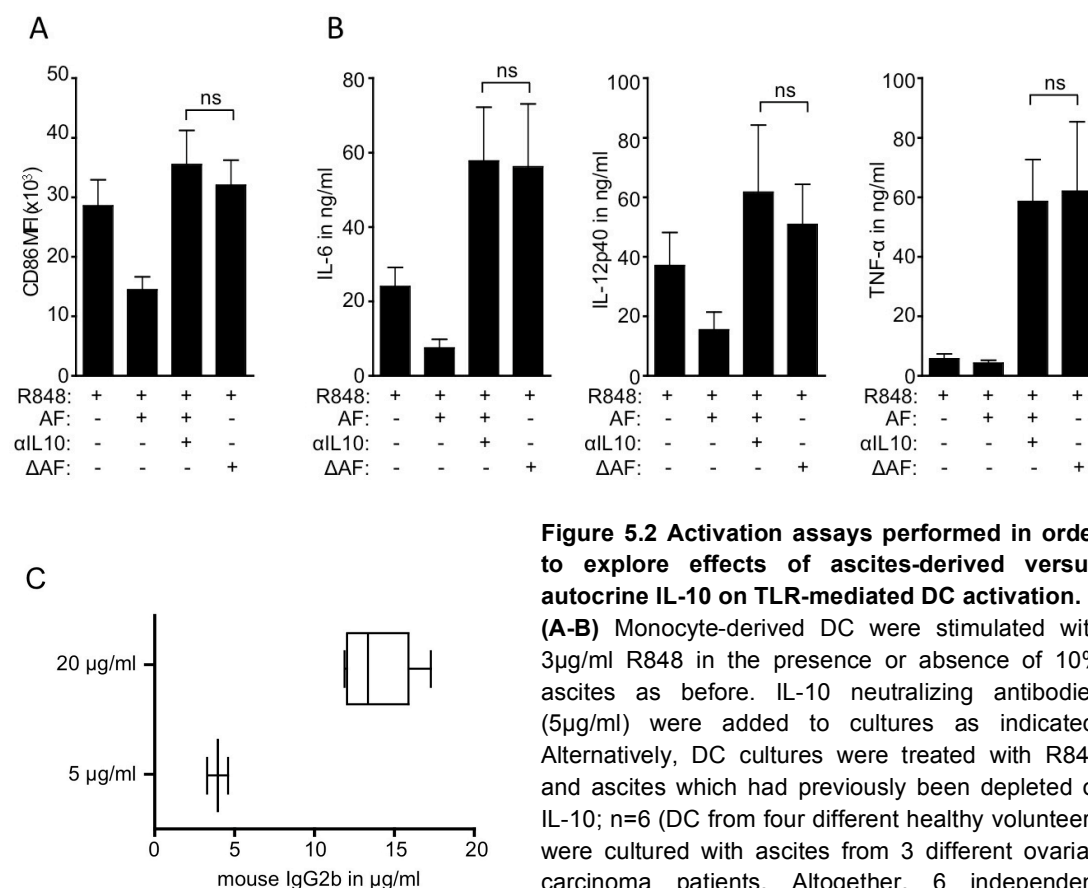


Figure 5.2 Activation assays performed in order to explore effects of ascites-derived versus autocrine IL-10 on TLR-mediated DC activation.

(A-B) Monocyte-derived DC were stimulated with 3µg/ml R848 in the presence or absence of 10% ascites as before. IL-10 neutralizing antibodies (5µg/ml) were added to cultures as indicated. Alternatively, DC cultures were treated with R848 and ascites which had previously been depleted of IL-10; n=6 (DC from four different healthy volunteers were cultured with ascites from 3 different ovarian carcinoma patients. Altogether, 6 independent experiments were performed. Cells from two volunteers were cultured with only one ascites sample each, while cells from the remaining two donors were cultured with two different ascites samples each). CD86 expression levels were measured by flow-cytometry, and cytokine levels were measured in cell culture supernatants by sandwich ELISA. One-way ANOVA (Friedman test with Dunn post test): ns = not significant. **(C)** Levels of IL-10 neutralizing antibody in ascites samples after attempted depletion of IL-10 using Protein G coated agarose beads. The depletion was attempted with 5 µg/ml (lower panel) neutralizing antibody in two samples (n=2) and with 20 µg/ml (top panel) neutralizing antibody in four samples (n=4).

However, it is of pivotal importance to note that under both culture conditions, the levels detected for all activation markers were considerably higher when compared to ‘baseline’ activation levels of R848 alone (Figure 5.2 A-B). This led us to discover a flaw in the design of the performed IL-10 depletion, and the pitfall we encountered is depicted schematically in Figure 5.3. The tumour environment in ovarian carcinoma harbours B lymphocytes (Nelson, 2010;Nielsen and Nelson, 2012;Nielsen et al., 2012), and the ascites samples extracted from within this environment are therefore certain to contain human immunoglobulins, possibly at high concentrations. Although the first step of the protocol was successful and IL-10 neutralizing antibody specifically bound to IL-10 in ascites, the agarose beads coated with Protein G added in the next step not only reacted with these antibody-antigen complexes, but rather, either due to higher affinity and/or higher concentrations of human immunoglobulins, mainly formed bonds with the latter. Due to preferential binding of human immunoglobulins, or, possibly, other proteins in ascites to the agarose beads, after centrifugation, the supernatant contained undesired IL-10 neutralizing antibody. Given the concentration of 20 µg/ml used in step 1, this residual antibody was most likely not saturated with IL-10. Hence, when this “depleted” supernatant was added to cell cultures, the IL-10 specific antibodies within had the capacity to also neutralize the autocrine IL-10 produced by monocyte-derived DC, explaining the elevated levels of activation in these samples when compared to ‘baseline’ levels of R848 alone.

Since IL-10 in the “depleted” ascites was bound to the neutralizing antibody, it was undetectable in the IL-10 specific ELISA that had been performed to verify the depletion, desceptively mimicking a successful removal of IL-10. To confirm the hypothesis that residual IL-10 specific antibody was present in the depleted ascites, we performed ELISA for detection of mouse IgG2b and indeed found high levels of IL-10 neutralizing antibody in ascites samples depleted with this method (Figure 5.2 C). The two samples from the pilot experiment depleted with a concentration of 5 µg/ml

α IL-10 antibody showed levels of approximately 4 μ g/ml, indicating that 80% of the antibody used had not bound to Protein G beads and remained in the supernatant after centrifugation. Among the four samples depleted using 20 μ g/ml, the IgG2b concentrations ranged from 13 – 17 μ g/ml. It is highly unlikely that the binding capacity of the Protein G coated beads had been exceeded at the used concentration of 20 μ g/ml IL-10 neutralizing antibody, because the amount of beads added had a total binding capacity of 2 mg/ml IgG (\approx 100-fold higher).

To summarize, these results confirmed that this approach was not successful, most likely due to direct competition of neutralizing antibody with proteins found in ascites, and a different experimental protocol had to be identified to achieve successful depletion of IL-10 from ascites.

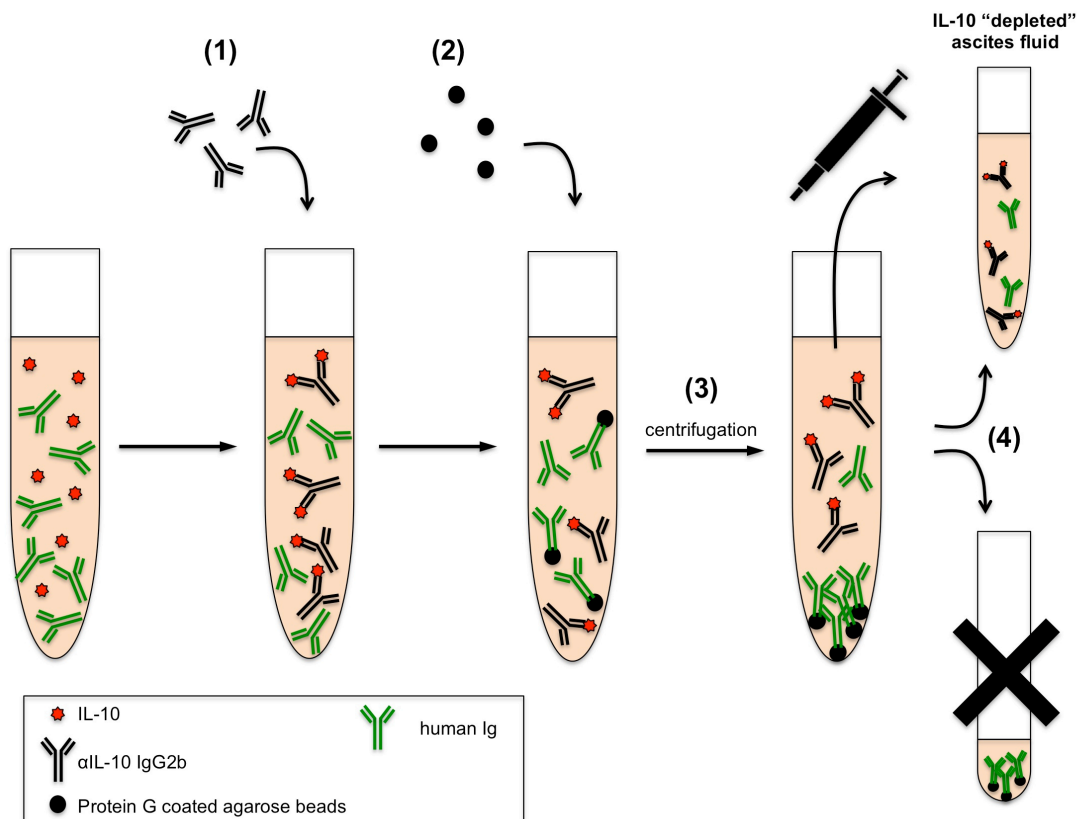


Figure 5.3 Schematic depiction of pitfalls encountered with the experimental approach using α IL-10 neutralizing antibody and Protein G coated agarose beads for depletion of IL-10 from ascites.

- (1) Ascites from ovarian carcinoma patients contains a high concentration of the patient's own, human immunoglobulins (green). Neutralizing α IL-10 IgG2b (black) is added to ascites at a concentration of 20 μ g/ml, successfully binding all IL-10 present in ascites.
- (2) Protein G coated agarose beads are added. For reasons of either higher affinity and/or higher concentration of human immunoglobulins in ascites, the antibody-antigen complexes consisting of mouse IgG2b and IL-10 are outcompeted by human immunoglobulins, which readily bind to the Protein G coated agarose beads.
- (3) Centrifugation creates a pellet of agarose beads with bound human immunoglobulins, leaving complexes of mouse IgG2b and IL-10 behind in suspension in the supernatant; the pellet is clearly visible at the bottom of the centrifuge tube.
- (4) The supernatant containing mouse IgG2b and IL-10 complexes is transferred into a fresh centrifuge tube. IL-10 cannot be detected by ELISA and this sample appears "depleted", because IL-10 is neutralized by its specific antibody. The α IL-10 antibody is, however, present at high concentrations and when this "depleted" ascites is added to cultures, autocrine IL-10 is neutralized.

5.2 Alternative methods for IL-10 depletion from ovarian carcinoma ascites and their drawbacks

The problems we encountered with our first approach to deplete IL-10 from ascites highlighted the difficulties one faces when working with a biological fluid containing an array of components that may affect experimental assays. Since no problems occurred with regards to the binding of IL-10 to the neutralizing antibody we used in the previous approach, we were content to use this same antibody in our next attempts. However, it had become clear that the removal of antibody-IL-10 complexes from ascites must be designed such that allows for specific elimination of these complexes only. If these attempts were high in their specificity, we would avoid competition with intrinsic components of ascites, making the chance of successful removal more likely. At the same time, such specific removal of antibody-IL-10 complexes would leave the other proteins in ascites unaffected, which was also highly desirable. With these aims in mind, we designed several approaches, the rationales and drawbacks of which are described in detail in the following sections.

5.2.1 Depletion of IL-10 from ascites using streptavidin beads and magnetic column separation

A schematic illustration of this experimental approach is shown in Figure 5.4. In order to achieve a specific removal of complexes formed between IL-10 and its neutralizing antibody (mouse IgG2b), we attempted to use a secondary anti-mouse IgG2b biotinylated antibody to target these complexes within the ascites sample. A subsequent addition of magnetic streptavidin beads would lead to a reaction with the biotinylated secondary antibody, leading to the formation of complexes between magnetic beads, secondary antibody and primary α IL-10 antibody with its bound ligand IL-10. By passing the sample over a magnetic column, we expected the

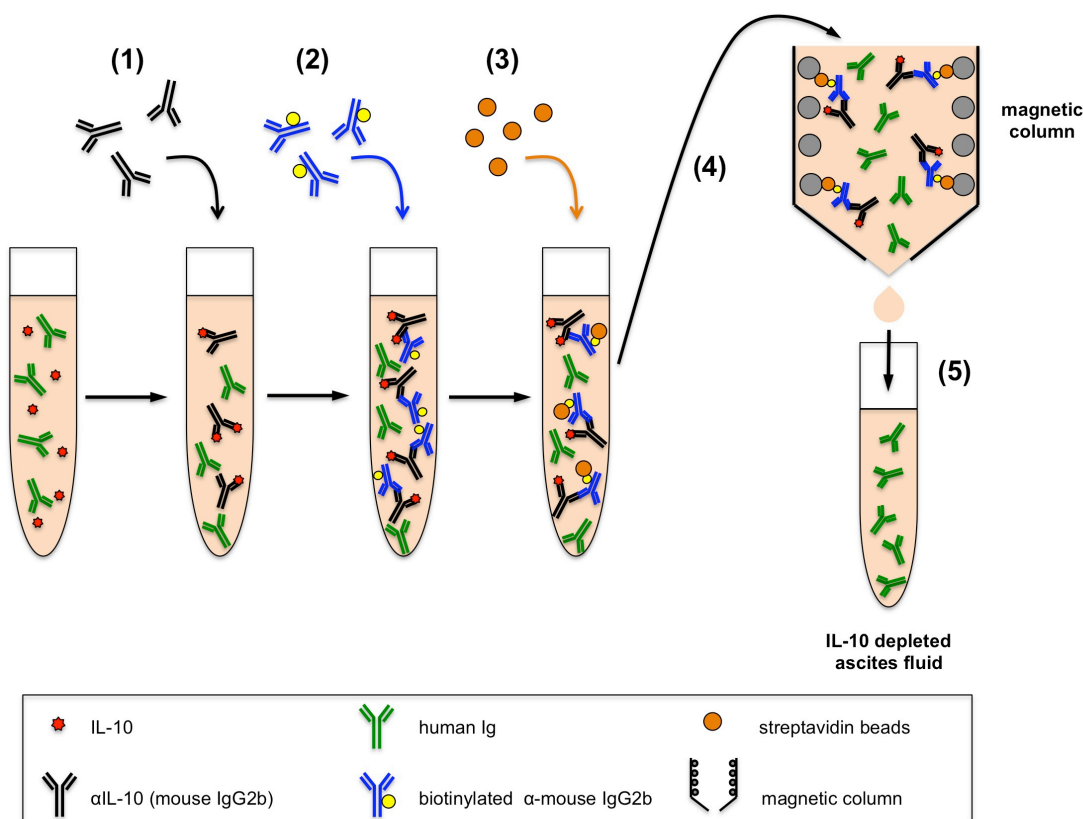


Figure 5.4 Schematic illustration of the experimental approach using streptavidin beads and magnetic column separation for depletion of IL-10 from ascites.

- (1) Neutralizing α IL-10 IgG2b (black) is added to ascites at a concentration of $10\mu\text{g/ml}$, binding all IL-10 present in ascites, forming antibody-antigen complexes
- (2) A secondary biotinylated α -mouse IgG2b antibody is added and binds to all complexes of α IL-10 antibody and α IL-10. The naturally occurring human Ig remain unaffected.
- (3) Streptavidin magnetic beads are added to the mixture, reacting with the biotin on the secondary α -mouse IgG2b antibody. Complexes consisting of streptavidin beads, biotinylated secondary antibody, and α IL-10 antibody bound to IL-10 are formed.
- (4) The sample is loaded onto a magnetic column, where magnetic streptavidin beads are retained together with both secondary antibody and primary α IL-10 antibody and its ligand IL-10.
- (5) The flow-through, now depleted of IL-10 but still containing its other components is collected in a fresh centrifuge tube.

magnetic streptavidin beads to be retained within the column together with its bound antibody-antibody-antigen complexes. The collected flow-through after passage over the column would now be depleted of IL-10 as well as the neutralizing antibody.

The concentration of α IL-10 antibody used in step 1 of this protocol was 10 μ g/ml. We chose this concentration because previous titration experiments as well as the technical data sheet for this antibody indicated that this concentration is abundantly sufficient to neutralize the levels of IL-10 found in the used ascites sample. Although 20 μ g/ml had been used previously (see above, section 5.1), this was considered unnecessary and the concentration was therefore adjusted to 10 μ g/ml in this experiment. To ensure binding of all primary antibody in step 2 of this protocol, we added the secondary biotinylated antibody at a concentration of 20 μ g/ml. Generous incubation times were provided in all steps of this protocol to allow the individual components to bind successfully. The streptavidin beads used (Miltenyi Biotec) are typically used for cell separation, and the amount required is therefore usually calibrated to the cell number processed. The amount we used in this experiment therefore had to be estimated but was rather generous in order to ensure binding of all secondary antibody, since this was crucial for the retention of antibody-antibody-antigen complexes within the magnetic column. We repeated step 4 of the protocol 3 times, i.e. the sample was passed over three fresh magnetic columns to ensure complete removal of complexes.

Although after each column passage the levels of α IL-10 antibody as detected by IgG2b ELISA decreased, residual antibody of 0.2 μ g/ml was present even after the last passage (Figure 5.5 A). Importantly, the secondary biotinylated antibody must have also been present, because the biotin interfered with the sandwich ELISA used to detect IL-10 levels in the depleted sample, creating an artifact of several-fold higher levels of IL-10 in the depleted samples compared to a non-depleted control (Figure 5.5 B). We therefore could not verify whether IL-10 was still present or not. A further pitfall of this protocol arose from the need to pre-moisten the magnetic columns used with PBS before loading of the sample. This led to a dilution of the

ascites sample, which could be observed as a fading of its natural yellowish colour, becoming increasingly less intense after each of the three column passages. This dilution could not be precisely quantified, and it would therefore be impossible to account for in future experiments when comparing with non-depleted ascites. The dilution phenomenon also led us to believe that the gradual decrease in α IL-10 antibody concentration (Figure 5.5 A) after each column passage may, in fact, not be the sign of successful removal of the antibody-antibody-antigen complexes, but may be in part an artefact caused by the dilution of the ascites samples with PBS.

For all these explained reasons, we deemed this approach not suitable for depletion of IL-10 from ascites samples and had to resort to alternative protocols.

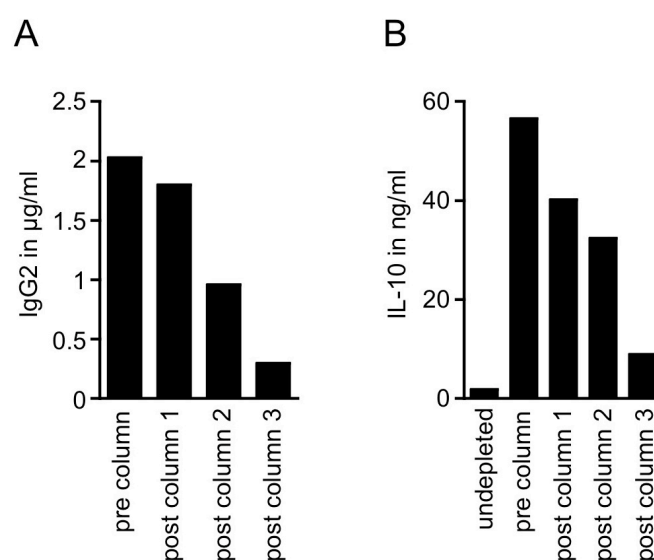


Figure 5.5 Attempted depletion of IL-10 from ascites using streptavidin magnetic beads and magnetic columns.

(A) Levels of IL-10 neutralizing antibody in ascites samples after attempted depletion of IL-10 using the protocol described in section 5.2.1. IgG2b levels were measured by ELISA either before passage of the ascites sample through magnetic columns ('pre column') or after one, two or three passages. **(B)** Results of IL-10 specific sandwich ELISA performed on ascites from this attempted depletion assay. IL-10 levels were measured after addition of IL-10 neutralizing antibody, secondary biotinylated antibody and streptavidin beads, and either before passage through magnetic columns ('pre column') or after one, two or three passages. In all samples, the levels of IL-10 appear considerably higher than in an undepleted control. This is due to an artefact of interference of biotinylated secondary antibody and/or streptavidin beads with our IL-10 ELISA. With increasing passages over magnetic columns, the measured values drop, indicating gradual removal of biotinylated antibody and/or streptavidin beads.

5.2.2 Depletion of IL-10 from ascites using magnetic Protein G beads pre-coated with IL-10 specific neutralizing antibody

Despite the problems we encountered with the use of Protein G coated agarose beads (see above, section 5.1), Protein G remained an agent worth considering for our purposes because of its very high affinity to immunoglobulins and its frequent use in antibody purification and immobilization protocols. We hypothesized that it had been the competition with other proteins present in ascites that led to unsatisfactory binding of neutralizing antibody to the beads, and therefore to its residue in the IL-10 depleted samples.

To avoid the direct competition for binding sites between neutralizing antibody and ascites-intrinsic proteins, in our next approach (Figure 5.6) we pre-coated magnetic Protein G beads with the neutralizing antibody before addition of such complexes to ascites samples. We expected that binding sites on Protein G beads that were already occupied by IL-10 neutralizing antibodies when encountering proteins in ascites would not be subject to competition. We hoped to achieve a binding of IL-10 to its neutralizing antibody that had been immobilized on the magnetic Protein G coated bead, and a subsequent removal of bead-antibody-antigen complexes by magnetic separation. The binding capacity of the used magnetic beads coated with Protein G was very high, and it was not feasible to saturate the beads using the IL-10 neutralizing antibody. However, we wanted to ensure that binding sites that remained vacant did not attract proteins from ascites, thereby altering its composition beyond IL-10 depletion. Therefore, after successful coating of beads with the neutralizing antibody (Figure 5.6, after step 1) and before addition to ascites (Figure 5.6, before step 2), we saturated the bead binding capacity using chromatographically purified unspecific mouse IgG (this step is not depicted in Figure 5.6).

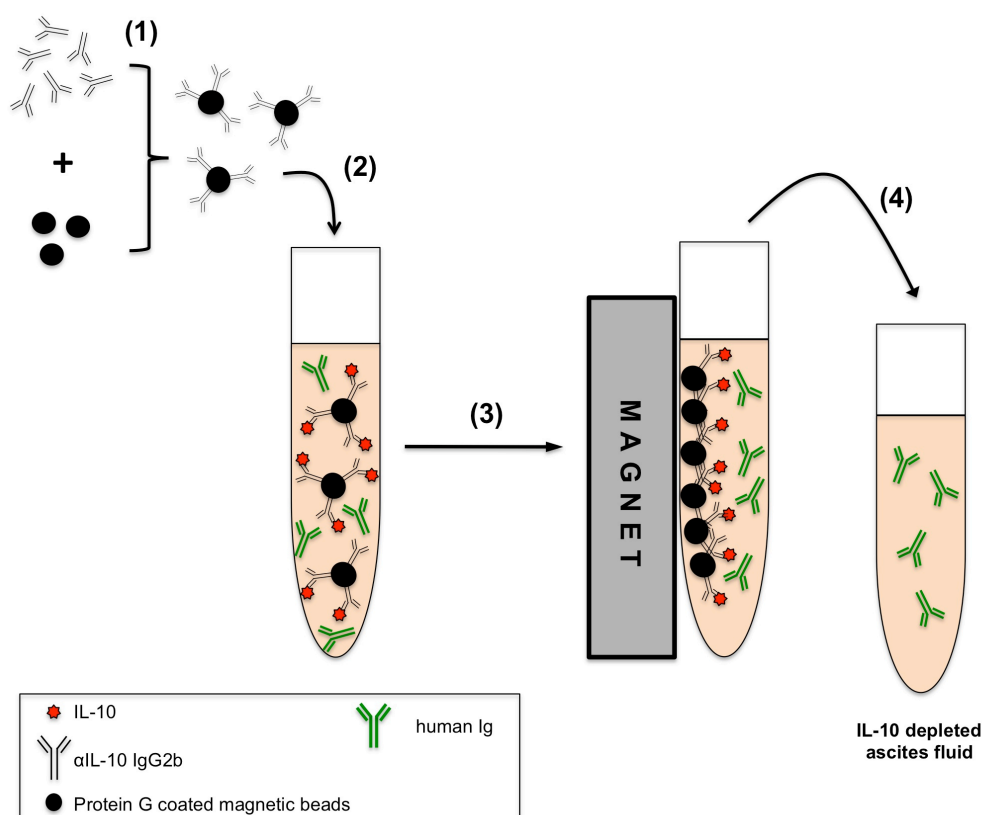


Figure 5.6 Schematic depiction of the rationale behind the experimental approach using magnetic Protein G beads pre-coated with neutralizing IL-10 antibody for depletion of IL-10 from ascites.

- (1) Protein G magnetic beads are incubated with IL-10 neutralizing antibody. The antibody binds to Protein G beads with high affinity.
- (2) The beads pre-coated with IL-10 neutralizing antibody are added to ascites and incubated to allow binding of IL-10 to its neutralizing antibody. The incubation times chosen were 1 hour or 16 hours overnight.
- (3) A powerful block magnet is used to remove complexes of beads, neutralizing antibody and IL-10 by retaining them at the wall of the centrifuge tube while...
- (4) the remaining, IL-10 depleted fluid is carefully transferred into a fresh centrifuge tube by pipetting.

In our first depletion attempt with this method, we incubated the ascites samples with the pre-coated beads overnight with continuous mixing (after step 2, before step 3). After removal of bead complexes (after step 4), we measured IgG2b levels in the depleted samples. The amount of neutralizing IL-10 antibody used to pre-coat the magnetic beads had been 10 μ g. Disappointingly, the detected levels of IL-10 neutralizing antibody in this sample reached the considerable level of 2 μ g (Figure 5.7), indicating that 20% of the starting amount of antibody must have detached itself from the beads during incubation. We suspected that this may have been due to the long overnight incubation time, and hence repeated the experiment, reducing the incubation to 1 hour. However, after this time, the levels of IgG detected in the ascites sample were even higher, reaching a level close to 10 μ g, which was almost 100% of the starting amount of neutralizing antibody. (Figure 5.7).

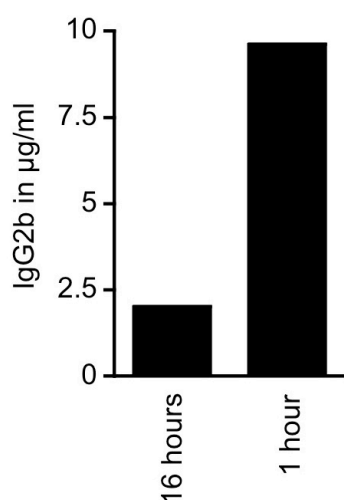


Figure 5.7 IgG2b levels in ascites samples after attempted depletion of IL-10 using magnetic Protein G beads pre-coated with IL-10 neutralizing antibody.

In two independent experiments, two different incubation times (16 hours overnight or 1 hour) were chosen. After both attempts, IgG2b was detected in high levels in ascites.

We concluded that evidently, even after pre-coating of Protein G beads with IL-10 neutralizing antibody before addition to ascites, the binding sites on the beads remain subject to competition. Due to higher affinity and/or concentration, neutralizing antibodies are outcompeted and displaced from their binding sites by proteins found in ascites.

This experimental approach has shown us that despite its high affinity to mouse IgG2b (the isotype of the neutralizing IL-10 antibody used) Protein G is not suitable for our purposes because it appears to bind human immunoglobulins and possibly other proteins in ascites with a superior affinity.

5.3 Successful depletion of IL-10 and PGE₂ from ascites using neutralizing antibodies covalently bound to NHS ester coated magnetic beads

After several failed attempts, we have identified a successful method for specific depletion of cytokines from ascites, using magnetic beads coated with NHS ester groups which have the ability to covalently bind antibodies. These beads are commercially available (Millipore), and the technical details of this method are described in Chapter 2 Material and Methods, section 2.11.4. The rationale closely resembles that of the aforementioned protocol which uses Protein G magnetic beads pre-coated with cytokine-specific neutralizing antibody (see above, section 5.2.2). However, an important difference is the nature of the bond formed between the magnetic bead and the neutralizing antibody. While the antibody binds to Protein G in a reversible way which can be easily disrupted e.g. in presence of another immunoglobulin with higher affinity to Protein G, the bond formed between NHS ester groups on the magnetic beads used in this protocol and free amine groups of the neutralizing antibody is covalent (Figure 5.8).

We first tested this method to deplete IL-10 from one ascites sample. When performing an IgG2b specific ELISA on the depleted sample, we could detect a concentration of 0.0013 µg/ml IL-10 neutralizing antibody (data not shown). The data sheet of this antibody provides titration guidelines suggesting that this concentration of antibody has no neutralizing ability. Additionally, if using this sample in cell culture in our usual protocol, it would only be added at 10% of the final well volume, and therefore further diluted by a factor of 10. We were therefore confident that this amount of neutralizing antibody would not neutralize autocrine IL-10 and was therefore negligible. The success of the depletion of IL-10 was further verified by IL-10 specific ELISA. The depleted sample showed IL-10 levels below the assay's sensitivity, while a mock-depleted control sample, using unspecific mouse IgG2b

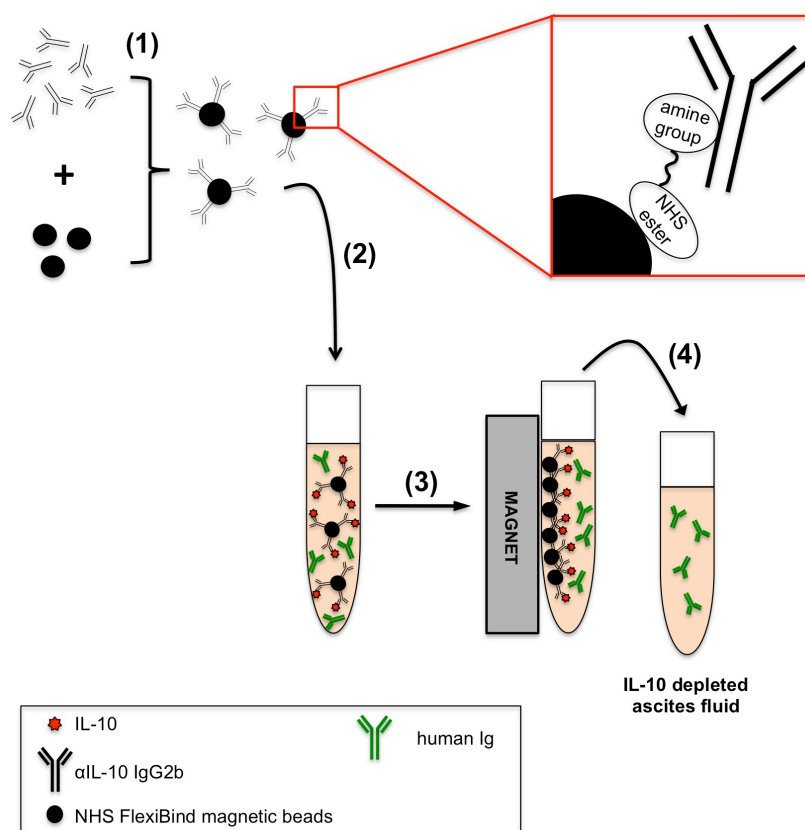


Figure 5.8 Schematic depiction of the experimental approach using magnetic NHS ester coated beads with neutralizing IL-10 antibody for depletion of IL-10 from ascites.

- (1) Magnetic NHS ester beads are coated with IL-10 neutralizing antibody. The bonds between the NHS ester group on the beads and free amines of the antibody are covalent. The covalent binding is achieved by following a carefully optimized protocol using specific buffers of defined composition and pH values.
- (2) Such pre-coated beads are added to ascites and incubated overnight to allow binding of IL-10 to its neutralizing antibody.
- (3) A powerful block magnet is used to remove complexes of beads, neutralizing antibody and IL-10 by retaining them at the wall of the centrifuge tube.
- (4) The remaining, IL-10 depleted fluid is carefully transferred into a fresh centrifuge tube by pipetting.

isotype control antibody, contained IL-10 at levels identical to the undepleted ascites (Figure 5.9).

Following this protocol, we depleted three further ascites samples from different patients of IL-10, verifying the success of this method by measuring IgG2b and IL-10 levels in the depleted samples each time. IgG2b levels were below 0.001 $\mu\text{g/ml}$ and IL-10 was not detectable in all depleted samples (data not shown).

As described in Chapter 4, we had identified PGE_2 as a further suppressive factor in ovarian carcinoma associated ascites acting alongside IL-10, specifically impairing $\text{TNF}\alpha$ production by monocyte-derived DC upon R848 stimulation. Although initial experiments did not suggest a contribution of autocrine PGE_2 to this suppression (see Chapter 4, Figure 4.7 D), PGE_2 can be produced by all cells of the body and we wanted to exclude the role of autocrine PGE_2 more robustly. Comparison of activation in cultures using ascites depleted of PGE_2 versus PGE_2 neutralizing antibody presented an elegant method to test this, and we therefore used the same depletion protocol as described above for IL-10 to deplete 2 ascites samples from ovarian carcinoma patients of PGE_2 . As with the IL-10 depletion, levels of PGE_2 specific neutralizing antibody in the depleted samples were in the range of 0.001 – 0.0013 $\mu\text{g/ml}$ and therefore negligible (data not shown).

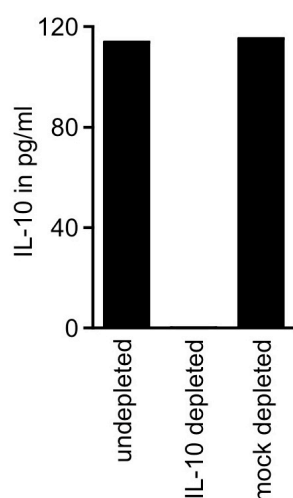


Figure 5.9 IL-10 levels in ascites after depletion using NHS magnetic beads.

IL-10 was measured by sandwich ELISA. While levels in the IL-10 depleted sample (middle) were below the sensitivity of the assay, levels in a mock depleted sample (right bar) using unspecific IgG2b isotype control antibody remained unaffected by the depletion procedure and were identical to the undepleted control (left bar).

PGE₂ removal from depleted samples was verified by PGE₂ specific ELISA. We saw a considerable decrease in PGE₂ levels in one of the depleted samples (Figure 5.10 A), indicating an almost complete removal of PGE₂ from ascites. The second ascites sample contained an approximately three-fold higher starting concentration of PGE₂, and here we observed PGE₂ levels reduced by 60% upon depletion (Figure 5.10 B). Importantly, in both samples, PGE₂ remained unaffected by mock depletion. In principle, the method was therefore successful and specific, but it appears that the antibody reached its saturation and could not bind all PGE₂ present. The protocol for depletion of PGE₂ therefore requires optimization in terms of the amount of α PGE₂ coated NHS beads used, or rounds of depletion that are necessary for complete protein removal.

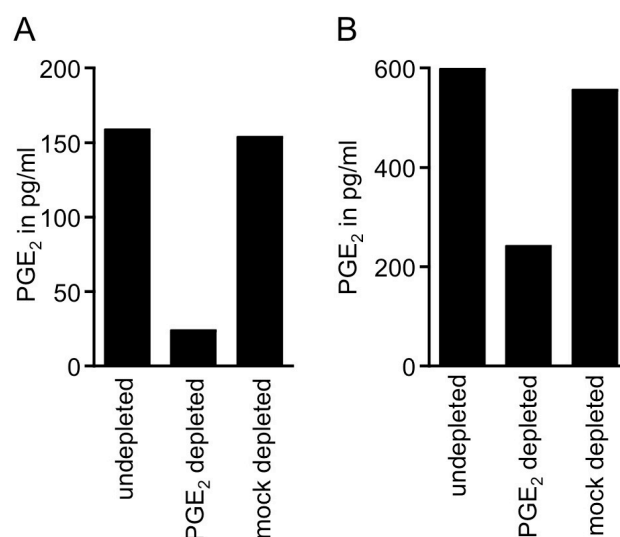


Figure 5.10 PGE₂ levels in ascites after depletion using NHS magnetic beads.

PGE₂ was measured by sandwich ELISA. Levels of PGE₂ were determined in undepleted, PGE₂ depleted and mock depleted ascites samples. One ascites sample is shown in A and B, respectively.

5.4 Initial experiments with IL-10 or PGE₂ depleted ovarian carcinoma ascites

Having at last succeeded in identifying a method which allowed us to specifically deplete either IL-10 or PGE₂ from ovarian carcinoma associated ascites, we conducted first experiments using these samples in order to elucidate the contribution of autocrine production of these two proteins to the observed suppression of TLR-mediated activation of monocyte-derived DC. It is important to note that this work is ongoing at present and the data shown in this section are therefore very preliminary.

We performed an activation assay using R848 as TLR stimulant, adding IL-10 depleted ascites from three patients to cell cultures at a final concentration of 10%. We included control samples using undepleted ascites from the same patients, also at a concentration of 10% with or without addition of IL-10 neutralizing antibody (5 µg/ml as previously). We observed no alleviation of suppression for CD86, IL-6 or IL-12 where IL-10 depleted ascites was added to cultures (Figure 5.11). This was also not observed for TNFα induction, but it is important to note that surprisingly, we did not see a suppression of TNFα in presence of ascites in this experiment (Figure 5.11, graph on the far right), and it is therefore not possible to conclusively interpret the influence of undepleted versus depleted ascites on TNFα in this instance. However, what is very clear in this assay is the striking impact that neutralization of autocrine IL-10 has on activation levels of monocyte-derived DC upon R848 stimulation. This is in accordance with our previous sets of data in this study using neutralizing IL-10 antibodies.

This assay was conducted before PGE₂ depletion could be verified by PGE₂-specific ELISA, and we were therefore not aware that removal of PGE₂ may have been incomplete. PGE₂ depleted samples had therefore also been included in this assay with their respective controls of undepleted ascites plus PGE₂ specific neutralizing antibody. However, because of the lack of suppression of TNFα in this experiment, these data were inconclusive (data not shown).

Further experiments with depleted ascites are necessary to elucidate the impact of autocrine IL-10 and PGE₂ on monocyte-derived DC activation in our experimental system. Having established a reliable method of specific depletion of the desired proteins from ascites, we hope to avoid further technical pitfalls while these important experiments are underway. The first experiment shown in Figure 5.11 points towards an overriding role of autocrine IL-10 in comparison to ascites-derived IL-10. However, these data require verification, and additional controls as well as further experimental procedures may in fact reveal more complex and intricate pathways regulating monocyte-derived DC activation in presence of ovarian carcinoma associated ascites. Our ongoing work and its rationale for dissecting the role of autocrine versus ascites-derived factors in our experimental scenario are put forward in more detail in the discussion to this chapter.

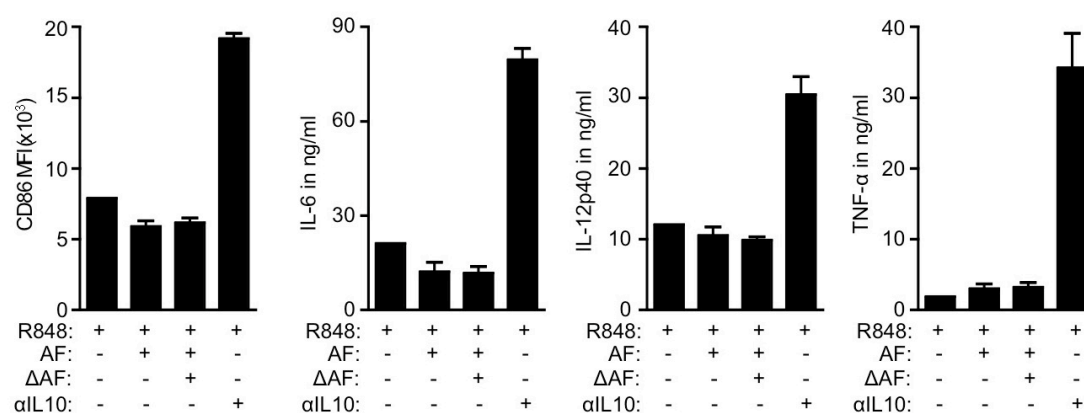


Figure 5.11 Initial activation assays performed with ascites that had been successfully depleted of IL-10 in order to explore effects of ascites-derived versus autocrine IL-10 on TLR-mediated DC activation.

Monocyte-derived DC were stimulated with 3μg/ml R848 in the presence or absence of 10% ascites. IL-10 neutralizing antibody (5 μg/ml) was added to cultures as indicated. Alternatively, DC cultures were treated with R848 and ascites which had previously been depleted of IL-10; For R848 n=1, for the other samples n=3 (1 experiment using monocyte-derived DC from one healthy control which were treated with ascites from three different ovarian carcinoma patients). CD86 expression levels were measured by flow-cytometry, and cytokine levels were measured in cell culture supernatants by sandwich ELISA.

Discussion

Ascites from ovarian carcinoma patients lends itself to research studies exploring the tumour environment in this disease, because its cellular composition as well as soluble factors within reflect to a great extent the constitution of the solid tumour mass. Its liquid form makes it a convenient material to study, since, if required, the soluble components can be separated from the cellular fraction by simple methods such as centrifugation and filtration, allowing for studies of its specific constituents. The experimental data presented in this chapter outline the difficulties we encountered when working with cell-free ascites and attempting its depletion of specific soluble factors.

Ascites is a biological fluid abundant with proteins, and their characterization has been the focus of several studies in the past, mostly with the aim to single out candidates that bear potential for clinical use as tumour biomarkers for diagnostic and disease monitoring purposes. A proteomic study of ovarian carcinoma associated ascites has identified more than 2500 proteins, 229 of which were found in the cell-free supernatant obtained after centrifugation, material identical to that used in our study (Gortzak-Uzan et al., 2008). These 229 proteins are likely to represent only the most abundant candidates, because the authors disclose the lack of detection of certain growth factors such as VEGF α and TGF β which are well documented as components of ovarian carcinoma ascites, and explain that these proteins were most likely missed in the proteomics analysis due to their relatively low abundance. Hence, it is only natural that the myriad of soluble factors in our ascites samples made a selective depletion of a particular protein very challenging, and as we have shown, in the protocol that finally led to the successful depletion of IL-10 and PGE₂, we had to take the composition of ascites into careful consideration.

The difficulties we encountered in our depletion attempts using protein G coated beads suggested the presence of ascites-associated proteins that competed for protein G binding sites with the α IL-10 neutralizing antibody used. Protein G is a streptococcal cell wall protein featuring binding domains for Fc and Fab fragments of immunoglobulins (Grubb et al., 1982; Kraulis et al., 1996). Because of this property, it is widely used for purification and immobilization of immunoglobulins for various applications. While IgG of numerous species including mouse and human IgG show high affinity for protein G, other human immunoglobulin subclasses such as IgA, IgD and IgM do not bind to protein G (Bjorck and Kronvall, 1984). The native molecule of protein G also features an albumin binding domain (Kraulis et al., 1996). The physiological concentration range of serum albumin is 3.5 – 5.0 g/dL, and as explained in the introduction to this chapter, its ascitic concentration in ovarian carcinoma is typically only slightly lower than in serum, resulting in considerable levels of albumin within the ascites. Due to its small size of 67 kilodalton (kDa), albumin easily passes through the filters used for filtration of our ascites samples and it was therefore present during depletion. However, interference of albumin with protein G was not a concern in our protocols, because the recombinant protein G used in our study has been modified such that it is devoid of its albumin binding domain. This modification is typically implemented on commercially available protein G with precisely this aim to circumvent undesired interference with albumin and ensure undisturbed binding of immunoglobulins (Akerstrom et al., 1987; Goward et al., 1990).

Given the adaptation of recombinant protein G as described above, the most likely components responsible for the difficulties we ran into in our protocols were human IgG molecules present in ascites. Human immunoglobulins are produced by plasma cells which arise from B lymphocytes after encountering antigens, receiving activation signals from helper T cells and being subjected to differentiation, enabling them to exert their effector function (Murphy et al., 2008). Circulating IgG levels in plasma are influenced by multiple factors such as age, gender and antigens encountered to date,

with values within the approximate range of 0.75 – 2 g/dL considered physiological (Stoop et al., 1969;Gonzalez-Quintela et al., 2008). IgG molecules migrate from the blood and diffuse into extravascular sites, and IgG has been detected in ascites of patients suffering from various pathologies, such as ovarian hyperstimulation syndrome (Abramov et al., 1999), peritoneal tuberculosis, bacterial peritonitis and different peritoneal malignancies including ovarian carcinoma (Abramov et al., 1999;Yildirim et al., 2002).

While diffusion from plasma is likely to be the most important source of IgG in ascites, it cannot be entirely excluded that more local processes of B cell activation and differentiation into plasma cells take place. Among the leukocyte populations infiltrating the tumour microenvironment of ovarian carcinoma, B lymphocytes are poorly studied. There is evidence suggesting their presence in ascites, although they are described as rather sparse in comparison with T cells, only constituting approximately 5-8% of the cellular infiltrate (Merogi et al., 1997;Melichar et al., 2001). However, rather than in suspension in ascitic fluid, B cells are likely to be present in surrounding tissue, in particular in the greater omentum, which in conditions of peritoneal carcinomatosis is typically abundant with immune aggregates, commonly referred to as ‘milky spots’. Perspectives on the importance of ‘milky spots’ in intraperitoneal immunity are controversial, with some reports regarding them as a dynamic form of secondary lymphoid tissue, referred to as omentum associated lymphoid tissue (OALT) (Shimotsuma et al., 1991;Heel and Hall, 1996). In this scenario, B cell differentiation into plasma cells and local immunoglobulin production is thought to take place in the omentum. Others support the notion that despite presence of immune cells including B and T lymphocytes in omental ‘milky spots’, these structures should not be classified as secondary lymphoid organs (Van Vugt et al., 1996;Yildirim et al., 2010). Several reports suggest that with their composition of adipose tissue, collagen, and abundant vasculature, ‘milky spots’ are likely to play an important role in metastasis formation due to the inclination of malignant cells to

adhere to these areas (Van Vugt et al., 1996;Gerber et al., 2006;Sorensen et al., 2009;Khan et al., 2010;Clark et al., 2013). Given the abundance of conflicting reports, the exact role of these omental aggregates remains somewhat enigmatic, and with particular regard to our study, it is impossible to assess the potential contribution of intraperitoneal B lymphocytes to IgG concentration within ascites in ovarian carcinoma.

As explained above, a limited number of studies have examined IgG levels in ascites in patients with various pathologies, but the data available is very sparse, and does not distinguish between peritoneal malignancies according to organ origin (Yildirim et al., 2002). In this regard, it is, however, important to note that even within a uniform patient group comprising only patients suffering from ovarian carcinoma, each patient's tumour tissue composition and immunological constitution is unique, and the concentration of IgG in different ascites samples is likely to be subject to large variations.

Despite no conclusive indication to date of exact IgG concentrations that might be expected to be found in our samples, we hypothesize that human IgG molecules in ascites were the deterrant in our attempts to deplete IL-10 with the use of protein G coated beads. It is likely that ascites derived IgG outcompeted the α IL-10 neutralizing antibody used for depletion in our study, occupying the majority of protein G binding sites and leaving the neutralizing antibody unbound and thus leading to residual α IL-10 antibody in depleted samples. This was the case both when α IL-10 antibody was added to ascites first and protein G coated agarose beads were added subsequently, as well as when protein G magnetic beads were pre-coated with the α IL-10 antibody before encountering the ascites components. The reasons for this outcompetition may have been either a considerably higher concentration of human IgG within ascites than mouse IgG used, or superior affinity of human IgG to protein G binding sites, or both.

The concentration of human IgG can be simply determined by ELISA, and in order to gain a better understanding of the difficulties we encountered, we plan to perform these measurements on undepleted samples and further on samples obtained from the attempts of IL-10 depletion by protein G coated beads. The concentration of human IgG in undepleted ascites samples will give us an indication of its ratio to the concentration of mouse α IL-10 IgG that we used. Assuming our hypothesis is correct and protein G binding sites during depletion were largely occupied by human IgG, a decrease of human IgG concentration in ascites samples after depletion should become apparent. Given the large binding capacity of the beads used in both attempts, this decrease may indeed be detectable by ELISA. Clarifying the reason for the failure of the depletion protocols using protein G coated beads will enable us to better understand the ascites composition and inform future studies using this material.

The attempt of cytokine depletion using streptavidin beads and magnetic columns posed many difficulties and was for a number of reasons not suitable for our purposes. Issues such as the necessity to calibrate magnetic columns with PBS leading to a dilution of ascites samples originated from the fact that magnetic column separation is typically used for cell enrichment where dilution of soluble components is of no concern. While it is often necessary to employ creativity in the laboratory when establishing new protocols, caution should be taken when straying far from the well established use of certain reagents and material for new applications.

Our initially unsuccessful attempts to deplete IL-10 from ascites finally led us to resort to the use of magnetic beads coated with NHS ester groups which form covalent bonds with free amine groups on the depleting α IL-10 antibody. With this protocol, we achieved successful removal of IL-10 from four ascites samples, and have shown that only negligible concentrations of residual neutralizing antibody are found in the depleted samples. We further extended this method to the use of specific α PGE₂ antibody, and have equally successfully shown that PGE₂ can be selectively depleted from ascites samples, although PGE₂ depletion was not complete where higher

concentrations were present, and further optimization regarding amount of antibody-coated beads and/or rounds of depletion is necessary. The established depletion protocol is likely to be applicable for the selective removal of a multitude of other ascites proteins for which specific antibodies are available.

Our preliminary data from experiments conducted with ascites depleted of IL-10 or PGE₂ need further confirmation and exploration. In the first experiment with depleted ascites samples as presented in this chapter, we did not observe a suppression of TNF α induction with malignant, non-depleted ascites. Comparison to samples with PGE₂ depleted ascites in this experiment was therefore to no avail, because these data could not inform about the contribution of autocrine versus ascites-derived PGE₂ to TNF α suppression which had been seen in our previous experiments. The remaining markers of activation were, however, suppressed in presence of ovarian carcinoma associated ascites, and we could therefore assess whether or not IL-10 depletion from the fluid alleviated this suppression. We used IL-10 depleted versus non-depleted ascites samples from three different ovarian carcinoma patients and tested their influence on R848-mediated activation of monocyte-derived DC from one donor only, hence these data are very limited and preliminary. However, they point towards a strongly overriding effect of autocrine IL-10 in the suppression of TLR-mediated up-regulation of CD86, and production of IL-6, IL-12p40 and even TNF α , because, as seen previously, addition of neutralizing α IL-10 antibody elevated the levels of all these markers far beyond the levels of samples cultured in presence of R848 only. Surprisingly, it appears that ascites-derived IL-10 does not have an impact on DC activation, since its depletion does not change levels of any activation markers. This is puzzling, because in our previous experiments as outlined in chapter 4, we saw a strong positive correlation between IL-10 levels in ascites and suppression of activation. It is unlikely that this correlation was entirely coincidental, but equally, given that depletion of ascites-derived IL-10 showed no relevance in our last experiments, it appears that IL-10 in ascites does not suppress TLR-mediated DC

activation in a direct manner. Studies of monocyte-derived DC in the field of autoimmunity and rheumatology have shown that presence of particular cytokines including IL-10 and IL-6 during DC maturation is necessary for the subsequent production of substantial amounts of autocrine IL-10 upon TLR activation (Torres-Aguilar et al., 2010; Hilkens and Isaacs, 2013). It is therefore feasible that ascites-derived IL-10 together with other factors found in ascites exert their suppressive function indirectly by leading to increased autocrine IL-10 production, and that such autocrine IL-10 is, in fact, the main agent directly responsible for the suppression of DC activation. However, we could not detect increased IL-10 production upon TLR activation in presence of ascites in our study (see chapter 3, Figure 3.6 C). These assumptions are therefore highly speculative, and nevertheless question the importance and contribution of ascites-derived IL-10 towards the observed ascites-induced suppression of DC activation. The regulation of cytokine production by DC is complex and comprises an intricate network of factors and receptors. The exact mechanisms behind the suppression of TLR-mediated activation of monocyte-derived DC as observed in our study remain unclear and require further clarification.

Regardless of the outcome of these further investigations, dissecting the role of autocrine versus ascites derived IL-10 and PGE₂ will provide a much more thorough insight into the mechanisms underlying the suppression of TLR-mediated activation of monocyte-derived DC than the use of neutralizing antibodies in cell cultures alone can contribute. This also applies to future studies of this kind where it may be of interest to elucidate autocrine immunoregulatory or –stimulatory cytokine networks. Distinguishing the differential influences of tumour-derived versus autocrine factors on DC and their activation with TLR agonists may constitute an important step towards a more thorough and profound understanding of the complex interactions within the tumour environment and may inspire more elaborate immunotherapeutic concepts in ovarian carcinoma.

Chapter 6

Discussion

In this study, we have investigated the influence of soluble factors within the ovarian carcinoma microenvironment on activation of DC by TLR agonists. Our findings have shown that the presence of ascites from patients suffering from advanced stage high-grade serous ovarian carcinoma impairs TLR-mediated activation of monocyte-derived DC, as observed by reduced up-regulation of the co-stimulatory molecule CD86 on the surface of DC, as well as decreased production of the cytokines IL-6, IL-12p40 and TNF α . Other markers of activation, such as surface molecules CD40, HLA-DR or PD-L1 and cytokines IL-1 β and IL-10 remained unaffected by ascites, pointing towards the selective alteration of certain pathways in monocyte-derived DC by factors within the ovarian carcinoma environment. Peritoneal fluid from patients with benign tumours of the ovary equally suppressed the TLR-mediated up-regulation of CD86 and production of IL-6 and IL-12p40. Intriguingly, TNF α levels remained unaltered in presence of benign fluid, suggesting that one or several factors present in ovarian carcinoma associated ascites with selective effect on TLR-mediated TNF α induction distinguish the malignant tumour environment from benign conditions.

With the use of specific neutralizing antibodies, we could identify the immunosuppressive cytokine IL-10 as an agent central to the observed suppression. Upon addition of α IL-10 antibody to cultures of monocyte-derived DC in presence of malignant ascites, activation by TLR7/8 agonist R848 and TLR4 agonist LPS was once more restored, and notably, the activation levels exceeded those achieved in presence of the respective TLR agonist alone. We further identified PGE₂ as a protein present in malignant ascites, and its selective neutralization alleviated the suppression of TNF α production by DC upon R848 stimulation. The specificity of TNF α

suppression by PGE₂ in ovarian carcinoma associated ascites was striking, with other activation markers remaining unchanged upon PGE₂ neutralization. PGE₂ was absent from peritoneal fluid of patients with benign ovarian tumours in our study, and given the increasingly suggested importance of eicosanoids and COX inhibitors in cancer immunology and immunotherapy, this protein may represent an intriguing candidate for further study in the context of ovarian carcinoma.

It is important to note, however, that even in the case of TNF α suppression, IL-10 was the central suppressive agent. The production of immunoregulatory factors including IL-10 by DC in order to prevent excessive immune activation is an important component of TLR stimulation. The fact that neutralization of IL-10 in cell cultures induced DC activation levels far beyond those promoted by TLR activation alone was a clear indication that such autocrine IL-10 was taking influence on DC and contributing to suppression of their activation. We felt prompted to explore the respective importance of ascites-derived and autocrine IL-10 in our experimental culture system. In pursuit of this scientific question, we have established an elegant method for selective depletion of IL-10 from ascites samples, using specific α IL-10 antibody covalently ligated to magnetic beads. As shown with the subsequent depletion of PGE₂ from ascites, this method is further applicable for removal of other proteins.

Our preliminary data with ascites devoid of IL-10 indicate that, in fact, depletion of this cytokine from ascites does not restore monocyte-derived DC activation by TLR7/8 agonist R848. These findings point towards an important role of autocrine IL-10 in our experimental system, however, as discussed in chapter 5, these results require further confirmation and clarification.

The need to understand the composition of the tumour environment and its effects on immunotherapeutic interventions is evident. In this regard, IL-10 represents a factor well documented in ovarian carcinoma by several previous studies, attributed an important role as a component of the immunosuppressive milieu with potential

correlation to disease stage and prognosis. Selective depletion of IL-10 as performed in our study may, however, reveal that despite its indisputable immunosuppressive properties, IL-10 in ovarian carcinoma ascites may not hinder future DC based vaccination strategies. In particular, data from pilot experiments with primary mDC in our study indicate that TLR-mediated activation of these cells may not be effected by ovarian carcinoma associated ascites. This suggests that IL-10 in the malignant tumour environment may not pose problems for immunotherapeutic strategies in terms of DC activation, but it is important to bear in mind that it may well effect other leukocyte subsets and nevertheless impede anti-tumour responses. A considerable decrease in systemic and peritoneal IL-10 levels after de-bulking surgery offers an attractive time frame for immunotherapeutic interventions while the potential effects of IL-10 are minimized.

Further experiments with IL-10 depleted ascites will elucidate the importance of autocrine and ascites-derived IL-10 produced upon TLR activation in more detail. Specifically, we are currently in the process of utilizing the molecular method of microarray analysis to gain insight into the signaling pathways that are affected in monocyte-derived DC upon TLR-activation in presence of IL-10 depleted versus non-depleted ovarian carcinoma associated ascites. Equally, fluid devoid of PGE₂ will be included in these sample cohorts, allowing for a better understanding of the mechanisms underlying the suppression of TNF α and showing whether other factors that had not been tested in our assays are effected by PGE₂. These experiments compliment the presented study excellently, because due to the wide scope of microarray analysis, gene expression patterns of a large range of activation parameters can be examined. In this regard, our study to date was certainly limited and it is well conceivable that many more aspects of TLR-mediated activation than those that we have analyzed are altered by presence of malignant ascites, and more specifically by IL-10 and/or other factors within. Not only the breadth of parameters examined will

provide unprecedented insights, the depth of gene expression pattern analysis can contribute substantially to a new understanding of our existing data.

A further line of work towards the completion of this study includes the verification of biological activity of the selective neutralizing antibodies used in our experiments. Specifically, assays to test the functionality of antibodies against IL-6, LIF, VEGF α and TGF β which showed no effect in our assays will be carried out, and the details of the experimental design for each of the used antibodies are in planning. Collectively, these additional experiments will refine the findings of our study, and allow for conclusive interpretation of our results thus far.

In light of unsatisfactory results achieved with currently available measures applied in the management and treatment of ovarian carcinoma, immunotherapy represents an exciting, relevant and promising approach to complement and enhance current therapeutic protocols. Within the field, DC vaccination strategies present an attractive line of work, pursued in many laboratories worldwide. Despite promising findings in preclinical studies, the translation of basic concepts into applied, clinical settings remains challenging. A major obstacle is posed by immunosuppressive factors within the tumour microenvironment, which can hinder anti-tumour immune responses by impairing DC activation. Findings presented in this study elucidate the processes that take effect when attempting to activate DC within the environment of ovarian carcinoma ascites. Further experiments with primary myeloid DC will be an important step towards the translation of our findings into more physiological settings. Equally, *in vivo* DC targeting studies in murine models of ovarian carcinoma represent an attractive option to expedite the field of DC immunotherapy and inform about the challenges posed by the microenvironment of malignant ovarian tumours in its entity and elucidate important new aspects that may require consideration in future design and application of vaccines relying on TLR-activation of DC subsets.

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